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Assessing Vascular Inflammation with Bioluminescence Imaging

Reece Joseph Goiffon
Washington University in St. Louis

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Assessing Vascular Inflammation with Bioluminescence Imaging

by

Reece Joseph Goiffon

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May 2015

St. Louis, Missouri
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<thead>
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ABI</td>
<td>Ankle brachial index</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass graft</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CAP</td>
<td>College of American Pathologists</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CHL</td>
<td>Cleveland HeartLab</td>
</tr>
<tr>
<td>CLIA</td>
<td>Clinical Laboratory Improvement Amendments</td>
</tr>
<tr>
<td>CMPC</td>
<td>Common myeloid progenitor cell</td>
</tr>
<tr>
<td>(c_pH)</td>
<td>Calculated pH</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>(\Delta FBS)</td>
<td>Heat-inactivated fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>GOx</td>
<td>Glucose oxidase, from \textit{Aspergillus niger}</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>MACE</td>
<td>Major adverse cardiovascular event</td>
</tr>
<tr>
<td>MAPS</td>
<td>MPO activity on a polymer surface</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>OLS</td>
<td>Ordinary least squares</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidized LDL</td>
</tr>
</tbody>
</table>

\(^1\)For a list of reagent and solution aliases, see the Appendix page 166
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCI</td>
<td>Percutaneous coronary intervention</td>
</tr>
<tr>
<td>Phox</td>
<td>Phagocytic NADPH oxidase</td>
</tr>
<tr>
<td>RBP</td>
<td>Reconstituted bovine plasma</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>WLS</td>
<td>Weighted least squares</td>
</tr>
</tbody>
</table>
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ABSTRACT OF THE DISSERTATION

Assessing Vascular Inflammation with Bioluminescence Imaging
by
Reece Joseph Goiffon
Doctor of Philosophy in Biology and Biomedical Sciences
(Molecular Cell Biology)
Washington University in St. Louis, 2015
Dr. David Piwnica-Worms, Chair
Dr. Erik Herzog, Co-Chair

Cardiovascular disease (CVD) is the leading cause of morbidity, mortality, and health care costs in the developed world and is often undiagnosed until clinical presentation with a major adverse cardiovascular event (MACE). Plasma myeloperoxidase (MPO) content is an emerging biomarker for risk, progression, and prognosis at different stages of CVD. Enzyme-linked immunosorbent assays (ELISAs) are currently used to measure clinical plasma MPO concentration, but ELISAs are costly and time-intensive. Luminol is a chemiluminescent compound with specificity for MPO activity in vivo, but is not sensitive enough for use as a bioluminescence reporter of plasma MPO oxidation. The luminol derivative L-012 is more sensitive to oxidation, but little is known about its sensitivity or specificity to reactive oxygen species (ROS) produced by MPO and its interactions with the myriad components of whole plasma. Therefore L-012 was first characterized as an MPO-dependent bioluminescence reporter in whole blood and plasma. The data indicated that L-012 is not a reporter of all MPO-dependent reactions, but specifically reports halogenation with chloride or bromide. Additionally, plasma components inhibited MPO both specifically and non-specifically and prevented precision measurement of plasma MPO activity with L-012 bioluminescence. To overcome this, a method was developed to isolate MPO on a solid substrate, remove small molecule antioxidants, and eliminate specific inhibition by plasma proteins. With this method,
MPO activity from human samples could be assayed using bovine plasma supplemented with purified human MPO as calibration standards. The assay can be performed in under an hour, comprises only two steps, and uses no costly immunologic reagents. The assay was validated with a pilot study using 72 plasma samples from cardiology patients undergoing elective catheterization. Individuals in this cohort assayed by the bioluminescence method were concordant with a parallel ELISA within $2 \pm 11$ µg/L MPO and overall the measurements from the two assays were not significantly different. To further validate the assay, an outside laboratory also measured MPO from 67 of the same plasma samples with an approved, clinical MPO ELISA. The newly developed MPO assay agreed with the outside ELISA on par with the parallel ELISA. In conclusion, a clinical assay for plasma MPO activity was developed with comparable sensitivity and specificity to current ELISAs which can be performed at a fraction of the time and cost.
There is a single light of science,

and to brighten it anywhere

is to brighten it everywhere.

ISAAC ASIMOV
1.1 Cardiovascular disease

1.1.1 Prevalence and risk factors

Cardiovascular disease (CVD) is the leading cause of morbidity, mortality, and health care costs in the United States and the rest of the developed world. The CVD mortality rate estimated for the United States in 2005 was 2,400 deaths per day. More than one of every three Americans has at least one form of CVD, and the lifetime risk after 40 in a previously disease-free individual is >50% for women and >65% for men. The correlation between economic development and CVD prevalence has led some to call it a “disease of affluence”, although lower socioeconomic populations in developed nations are most afflicted. CVD burden is rising in developing economies as well, attributable to sedentary lifestyle, decreased infectious disease mortality, urban environmental exposures, and shifts in dietary norms. While developed nations are projected to experience decreasing losses in disability-adjusted life years due to CVD through 2020, developing nations will see 55% greater loss in the same period. By 2020, CVD is expected to be the leading cause of death globally, with the heaviest burden shifting to developing nations.
CVD risk can be predicted by both acquired and inheritable traits. Associations with behaviors and their effects have led to CVD being considered a lifestyle disease. Tobacco use and physical inactivity are drivers of acquired CVD progression, as are dietary factors such as high sodium, high trans-unsaturated fatty acid, and low poly-unsaturated fatty acid intake. Risk is also heritable: diagnosis of premature CVD in parents or grandparents increases the odds of CVD development by a factor of ~2 or more after adjusting for lifestyle risk factors. Number of siblings and the extent of their disease also predicts risk of future CVD development. Propensity for many secondary lifestyle risk factors, such as elevated body mass index, abdominal fat, low-density lipoprotein (LDL), total cholesterol, and low high-density lipoprotein (HDL) can also be considered heritable risk factors. Although molecular biology techniques have helped advance research, specific CVD genetic markers have yet to outperform traditional risk factors.

The greatest danger of progressive CVD is a major adverse cardiovascular event (MACE). The definition of MACEs varies slightly between studies, but generally includes myocardial infarction (MI), stroke, and acute coronary syndrome (ACS). Most MACEs are caused by thromboemboli obstructing blood flow in vulnerable arteries: coronary obstruction causes ACS and eventually MI, cerebral obstruction results in stroke. These arterial systems are poorly supported by anastomoses, experience high systolic blood pressure, and supply vital and energetically demanding organs. Nearly every MACE can be attributed to atherosclerosis as the initiator of thrombosis.

1.1.2 Historical perspective of atherosclerosis

Atherosclerosis is pathological inflammation in the arterial wall, creating asymmetric loci of thickened intima called atheromas or plaques. Early-stage atherosclerosis in central arteries
Chapter 1: Introduction

Cardiovascular disease

appears in childhood and is nearly universal.\textsuperscript{22–24} These fatty streaks can progress through life and become vulnerable plaques, which tend to have thinner fibrous caps, eroded shoulder regions, calcifications, and necrotic cores. Consensus on the mechanisms and impact of atherosclerosis have changed radically throughout medical history.

Atherosclerosis has occurred in humans for at least 5,000 years.\textsuperscript{25} Although it was described by anatomists as early as the 16\textsuperscript{th} century, it was not given its own nomenclature until 1829.\textsuperscript{26,27} Through the remainder of the 19\textsuperscript{th} century, atherosclerosis was included in the then-mainstream “senescence hypothesis” that diseases of aging were part of normal physiology. Early investigations suggested that inflammation was involved, but pathologists debated if this was a cause or consequence.\textsuperscript{28} In 1913, a military researcher in St. Petersburg began to publish on induced atherosclerosis in rabbits fed high-cholesterol diets. The work would remain largely ignored by the medical community until 1950; from then it took more than a decade for the cholesterol hypothesis to be accepted.\textsuperscript{29–31} Research shifted back to inflammation in a similar manner, but has only been fully accepted as the driver of atherosclerosis in the last 20 years.\textsuperscript{32–37} Researchers are now probing the cellular mechanisms of plaque progression by considering the interactions of hemodynamic stress, lipid metabolism, and the immune system.

1.1.3 Innate immunity and atherosclerosis

Monocyte-lineage cells are known to be central components of atherosclerosis progression.\textsuperscript{38} Monocytes differentiate from a common myeloid progenitor cell (CMPC) shared with granulocytes and platelets. As CMPCs mature into monocytes, they can be categorized based on their expression of Ly-6C as “resident” Ly-6C\textsuperscript{low} or “inflammatory” Ly-6C\textsuperscript{high} monocytes, the latter of which is implicated in atherogenesis.\textsuperscript{39} These Ly-6C\textsuperscript{high} monocytes also express
myeloperoxidase, which contributes to oxidative damage.\textsuperscript{40} (see page 10) Depleting monocytes in a murine atherosclerosis model markedly reduces lesion initiation and development, but established plaque inflammation and rupture is not affected.\textsuperscript{41} This suggests that monocytes play a key role in establishing new plaques in this model, but progression is driven by other cells. Some of the candidate cells reside in the vascular wall and are derived from monocytic precursors.

Ly-6C\textsuperscript{high} monocytes can further differentiate into two classes of antigen-presenting cells (APCs) abundant in plaques. Macrophages are long-lived APCs that secrete cytokines and process lipids in atheromas.\textsuperscript{42,43} Scavenger receptors on their surface recognize LDL and oxidized LDL (oxLDL) for uptake. LDL and oxLDL accumulate to cytotoxic concentrations, differentiating the macrophages into foam cells that form the necrotic cores in vulnerable plaques. Dendritic cells (DCs) are a diverse class of APCs, some of which are also derived from Ly-6C\textsuperscript{high} monocytes.\textsuperscript{44,45} Monocyte-derived DCs are thought to promote plaque progression similarly to macrophages, while evidence suggests DCs derived directly from CMPCs may inhibit atherogenesis.\textsuperscript{46–49} The heterogeneity between and within the two classes of APCs suggests that the cells are better described along a continuum of effector molecule expression that would more closely define their roles in atherosclerosis.\textsuperscript{50,51}

Another group of innate immune cells derived from CMPCs comprises granulocyte lineages that differ in function from macrophages and DCs. Neutrophils and their immature, banded forms compose ~95% of circulating granulocytes in healthy adults; the remaining granulocytes are almost entirely eosinophils with a small fraction (~1%) basophils.\textsuperscript{52} Neutrophils are phagocytes, much like monocytes and macrophages, and primarily function as first-line antimicrobial cells. Unlike the chronic inflammatory APCs, unperturbed neutrophils are cleared from circulation in ~5 days and die within hours once activated by inflammatory
mediators or foreign antigens. Neutrophils are armed with granules packed with an array of hydrolases and oxidoreductases to kill invading microbes, which can be done by fusing granules to phagosomes or by expelling the enzymes into the extracellular space along with genomic DNA in aptly-named neutrophil extracellular traps (NETs). More recent findings implicate neutrophils in animal and human atherosclerosis through diverse mechanisms, including endothelial disruption, leukocyte recruitment, plaque destabilization, cytokine production, and metabolite modification. Myeloperoxidase is also concentrated in primary granules and has numerous atherogenic effects on disease models. Together, this evidence suggests that neutrophils may have a role comparable to that of macrophages in the transition from stable to vulnerable plaques and the subsequent MACEs that result from plaque rupture. Differentiating stable and vulnerable lesions is an active area of CVD research.

1.1.4 Assessing plaque vulnerability

The term “vulnerable plaque” was first introduced in 1989 to specify lesions that were prone to endothelial ruptures and subsequent thrombus formation. This was a departure from the belief that ACS resulted from coronary artery occlusion by large atheromas; vulnerable plaques are not necessarily large enough to perturb blood flow prior to rupture. In one study, new MACEs in patients previously treated with percutaneous coronary intervention (PCI) often had culprit lesions that were mild or unnoticed at the time of the first intervention. This study also showed that predicting future MACEs from lesions uninvolved in an initial MACE was difficult without invasive assessments of plaque morphology.

Plaques can be classified based on lipid content, fibrous cap structure, calcifications, and cellular infiltrates. Ruptured plaques are responsible for most MACEs and are richly
infiltrated with inflammatory cells. (see page 4) The luminal thrombus generally extends from the point of endothelial rupture into the plaque core containing necrotic debris, foam cells, extracellular lipids, fibroblasts, and calcifications. In contrast, eroded plaques are typified by a lack of endothelium over a larger section of the artery and a small or absent lipid core. The exposed intima is primarily smooth muscle cells with inflammatory infiltrates ranging from absent to moderate.\textsuperscript{64–66} Thrombi are thought to form more slowly on erosions than on ruptured plaques, but are more likely to embolize. Calcified nodules are the rarest of the three thrombotic plaques and are defined by calcifications that erupt through the endothelium and induce thrombosis in the arterial lumen. These are more common in the right coronary artery and are thought to be the result of repeated plaque exacerbation and healing in vessels subject to torsional stress.\textsuperscript{67,68}

Plaque vulnerability is difficult to measure via traditional angiography, but other techniques have been developed with improved resolution, tissue penetration, and molecular imaging capabilities. Computed tomographic angiography and magnetic resonance imaging (MRI) can detect lesions invisible to traditional angiography with better resolution and contrast.\textsuperscript{69} Molecular MRI probes have also been developed to target processes behind plaque vulnerability and thrombosis such as platelet activation, neovascularization, leukocyte adhesion, and foam cell apoptosis.\textsuperscript{70} Positron emission tomography and single photon emission tomography have comparably poor spatial resolution, but can be extremely sensitive for molecular imaging.\textsuperscript{71,72} These techniques vary in advantages and disadvantages, but are all limited to macroscopic resolution. The underlying structures that impart vulnerability can be microscopic and require intravascular techniques to visualize. High-resolution imaging of individual plaques, their underlying tissue organization, and some molecular processes is possible with intravascular ultrasound, optical coherence tomography, and intravascular
fluorescence imaging. This allows physicians to assess a patient’s atherosclerosis on a plaque-by-plaque basis, but with associated risks and costs that prohibit widespread use.

1.1.5 Vulnerable patient biomarkers

Secondary prevention is treatment of subclinical conditions before substantial morbidity. As CVD often first presents with MACEs, prioritizing pre-clinical disease based on statistical indicators of plaque vulnerability is a favorable strategy. This approach focuses on better screening techniques to identify “vulnerable patients” rather than invasive imaging for vulnerable plaques. Vulnerable patients are defined by systemic characteristics with tests that are accessible, minimally invasive, cost effective, and outperform current assessments such as Framingham Risk Score. Poor sensitivity from current screening methods has led researchers to consider direct measures of inflammation, myocardial damage, and microvascular dysfunction. Measuring plasma biomarkers for these pathologies requires only a blood draw and clinical laboratory equipment. Of note, the gold standards for MI diagnosis are plasma markers—the cardiac troponins—with which the assay receiver operating characteristic curve integral can be >0.90 with modern high-sensitivity kits. Many other biomarkers for aspects of CVD are in use or are being investigated for clinical utility. (Table 1.1) Parameters approximated by these biomarkers include systemic and vascular inflammation, predisposition for blood coagulation, myocardial ischemia, and hemodynamic strain. Most are circulating proteins that are assayed with immunologic purification techniques such as the enzyme-linked immunosorbent assay (ELISA). A typical sandwich ELISA uses animal antibodies conjugated to enzymes such as horseradish peroxidase (HRP) to produce quantifiable changes in a chemical reporter. ELISA costs are high as they use many biological reagents and are labor-intensive to perform. A promising biomarker, myeloperoxidase (MPO), is capable of
Table 1.1: Current and potential plasma biomarkers for CVD risk stratification and diagnosis.\textsuperscript{81–84} Most are proteins measured with clinical immunoassays such as ELISA.

<table>
<thead>
<tr>
<th>Indication</th>
<th>Analyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>cardiac injury</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td></td>
<td>carbonic anhydrase III</td>
</tr>
<tr>
<td></td>
<td>creatine kinase-MB (activity)</td>
</tr>
<tr>
<td></td>
<td>creatine kinase-MB (mass)</td>
</tr>
<tr>
<td></td>
<td>glycogen phosphorylase BB</td>
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<tr>
<td></td>
<td>heart fatty acid binding protein</td>
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<td></td>
<td>hydroxybutyrate dehydrogenase</td>
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<td></td>
<td>troponin I</td>
</tr>
<tr>
<td></td>
<td>troponin T</td>
</tr>
<tr>
<td>congestive heart failure</td>
<td>B-type natriuretic peptide</td>
</tr>
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the same reaction achieved with HRP in clinical ELISA kits; this presents a new possibility in secondary CVD prevention.
1.2 Myeloperoxidase

1.2.1 Structure and function

Myeloperoxidase (MPO) was first isolated from purulent fluids taken from tuberculosis patients. Initially named “verdoperoxidase” for the green color it gave to pus, MPO received its modern name once it was discovered to originate from myeloid-lineage cells. Human MPO is encoded by an 11 kb gene on chromosome 17q23.1 near the other anti-microbial human peroxidases with which it shares a genetic common ancestor: lactoperoxidase and eosinophil peroxidase. MPO is transcribed as preproMPO and contains a signal peptide sequence targeting it to the endoplasmic reticulum (ER), where it is heavily glycosylated with mannose-rich oligosaccharides and receives a heme cofactor. The 89 kDa proMPO then arrives to the cis-Golgi, from which it may have two different fates. Most loses the pro-peptide in the trans-Golgi and is sent to primary granules for endoproteolysis and dimerization into tetrameric, 150 kDa mature MPO. (Figure 1.1) Some proMPO instead receives further glycosylation with complex oligosaccharides in the cis-Golgi while retaining the pro-peptide for constitutive secretion as a 89 or 84 kDa monomer. Although mature MPO is a tetramer, each symmetric dimer functions independently. Additionally, the active site is not structurally modified during Golgi processing, suggesting that the secreted monomeric form may be similarly active as the mature form. Isoforms of mature MPO with modified subunits and enzymatic properties have been isolated from neutrophils, but little is known about the biological importance of these variants.

MPO is a member of the reactive oxygen species (ROS) cascade important in human innate immunity. (Figure 1.2) Defects in the phagocytic NADPH oxidase (Phox) complex that initiates the ROS cascade manifest as chronic granulomatous disease (CGD), characterized
Figure 1.1: Crystal structure of mature MPO. preproMPO is translated from a single gene product cleaved to proMPO in the ER. As it progresses through the Golgi network, proMPO is bound to a heme cofactor, glycosylated, and cleaved into a heavy and light chain (red and blue, respectively) linked by disulfide bonds. Tetrameric MPO is formed in the primary granules by removal of the pro-peptide and formation of covalent disulfide bonds between two heavy chains. The reaction centers (Fe$^{3+}$ of the heme shown in orange) of each dimer function independently. A small portion of proMPO is processed as a single, intact peptide and constitutively secreted as an active monomer. Structural data from Fiedler.\textsuperscript{102}

by frequent and severe microbial infections.\textsuperscript{103} The Phox complex generates superoxide ($O_2^-$) in the phagosomal space, which is quickly dismutated into hydrogen peroxide ($H_2O_2$). MPO catalyzes the reaction between $H_2O_2$ and Cl$^-$ to produce hypochlorous acid (HOCl), the active ingredient in household bleach.\textsuperscript{104} Neutrophils initiate this reaction by fusing their primary, or azurophilic, granules to phagosomes where $H_2O_2$ is being produced. Although these granules contain other antimicrobial enzymes, MPO is the most abundant and constitutes up to 5% of the mature neutrophil dry weight.\textsuperscript{57,105} MPO genetic deficiency does not manifest as CGD,
Figure 1.2: MPO and the phagocytic ROS cascade MPO normally functions as part of the neutrophil ROS cascade. Phosphorylation events cause the assembly of the Phox complex, which transfers electrons from cytosolic NADPH to molecular oxygen in the phagosome, creating superoxide (O$_2^-$) radicals. O$_2^-$ is dismutated to H$_2$O$_2$ in acidic environments such as the phagosome or in the presence of superoxide dismutase. H$_2$O$_2$ can directly oxidize biomolecules or serve as a substrate for MPO to produce HOCl. Unlike H$_2$O$_2$, HOCl cannot be neutralized by defensive catalases excreted by phagocytosed microbes although there is some evidence suggesting that neutrophils with dysfunctional MPO have increased phagocytosis and Phox activation.$^{106-109}$ Clinical presentation of MPO deficiency is most common in diabetics with compromised immune systems, but has been known to manifest in patients with disseminated Candida infections.$^{110,111}$ Genetic screening in the United States, Germany, and Italy suggests that only 0.05–0.15% of the population has MPO mutations manifesting as defects in protein sequence or processing, and in only a small fraction of these individuals will a mutation become symptomatic.$^{112-114}$

MPO reaction kinetics are complex owing to the flexible redox chemistry of the heme cofactor. Native MPO contains Fe$^{3+}$ with no coordinated oxygen, but heme oxidation state and ligand binding result in five intermediate states. (Figure 1.3) H$_2$O$_2$ generated by the Phox complex or oxidative metabolism oxidizes native MPO twice to form MPO compound I. Physiologic Cl$^-$ can donate two electrons to reduce compound I back to native MPO, forming HOCl as a byproduct. This two-electron redox cycling is known as the halogenation cycle and can utilize electron donors Br$^-$, I$^-$, or the pseudohalide SCN$^-$ in addition to Cl$^-$.}$^{115}$
Figure 1.3: MPO redox chemistry. The current model of MPO enzymatic activity includes six redox and ligand-binding states for each catalytic heme center. $\text{H}_2\text{O}_2$ (red) generated by the Phox complex or oxidative metabolism serves as a dual electron acceptor to oxidize MPO to compound I. Two electrons are transferred from abundant halide ions to compound I to form hypohalous acids, primarily HOCl \textit{in vivo}, which are responsible for MPO-mediated microbial killing. This halogenation cycle of two-electron transfers is rapid, but MPO can be shifted into slower peroxidation cycles by single electron transfers from donor species AH$_2$. Buildup of radical peroxidation intermediates AH$^\cdot$ along with excess $\text{H}_2\text{O}_2$ favors the shift of MPO into peroxidation activity, kinetically sequestering MPO from halogenation. In this manner, $\text{H}_2\text{O}_2$ serves as both a reactant and inhibitor of MPO-mediated halogenation. Scheme adapted from Malle, Furtmüller, Sattler & Obinger.\textsuperscript{115}  

However, compound I can also oxidize organic molecules in a single-electron reaction to form MPO compound II. In the presence of excess $\text{H}_2\text{O}_2$, compound II can enter a slow, catalase-like cycle with compound III and ferrous MPO, shifting enzyme mass away from the halogenation cycle. In this way, $\text{H}_2\text{O}_2$ is both a substrate for- and an inhibitor of MPO halogenation.\textsuperscript{116} Accumulating radicals produced by peroxidation can also react with native MPO to form the ferrous intermediate, further shifting activity away from halogenation. This feedback inhibition loop manifests as an initial kinetic burst of $\text{H}_2\text{O}_2$ consumption followed by steady-state kinetics.\textsuperscript{117} This shift can be influenced by pH, with optimum halogenation kinetics found in acidic environments similar to the lysosome.\textsuperscript{118} Substrate (pseudo)halides also influence redox cycle preference with Cl$^-$, Br$^-$, I$^-$, and SCN$^-$ increasingly favoring halogenation over peroxidation.\textsuperscript{118–120}
1.2.2 Pathology

MPO and its product ROS are not able to distinguish biomolecules originating from the host from those made by invading microbes. Oxidation of host biomolecules has a number of negative consequences that have been observed experimentally in vitro and naturally in vivo. Genetic polymorphisms causing increased MPO production have been associated with early-onset multiple sclerosis, but some animal and human studies paradoxically suggest that the hypomorphic or downregulated MPO may promote other mechanisms of disease development. Genetic associations with Alzheimer’s disease have also been conflicting, but MPO has been shown to associate with amyloid plaques along with apolipoprotein E.\textsuperscript{121}

MPO can target alter nucleic acids either by peroxidation or halogenation, so it is not surprising that researchers have found links between MPO and neoplasms. Cells producing MPO may be subject to oxidative damage if inhibition mechanisms become dysfunctional. Certain point-mutations in the MPO promoter region are associated with acute promyelocytic leukemia, and circulating primary tumor cells with these mutations have increased MPO mRNA levels.\textsuperscript{122} Expression and genotype studies have also associated MPO with solid tissue tumors such as cancer of the colon, breast, and pancreas.\textsuperscript{123–125}

CVD is the chronic disease most associated with pathologic MPO activity. In addition to neutrophils (see page 5), macrophages in atheromas also express MPO.\textsuperscript{126} Human MPO expressed in macrophages transplanted into a murine atherosclerosis model resulted in increased lesion size, suggesting that the enzyme has some causative role.\textsuperscript{127} Part of the explanation focuses on molecules oxidation products which have been shown to increase lesion progression. MPO can directly activate some matrix metalloproteinases (MMPs), which contribute to plaque progression and fibrous cap erosion.\textsuperscript{128} This effect can be compounded indirectly by oxidative inactivation of MMP inhibitors.\textsuperscript{129} LDL can be converted to into
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atherogenic oxLDL by ROS produced by MPO. oxLDL is recognized by scavenger receptors on the surface of macrophages and accelerates differentiation into foam cells. oxLDL is similarly oxidized and rendered dysfunctional by MPO; this inhibits reverse cholesterol transport by which HDL normally protects against atherogenesis. MPO disrupts nitric oxide signaling and reduces vasomotor activity in response to coronary ischemia. The reactive nitrogen species created by MPO are capable of modifying amino acids, perhaps by transient nitrite intermediates at sites of nitric oxide production, providing another MPO-mediated route to dysfunctional proteins. Lastly, MPO can induce endothelial cell apoptosis and exposure of underlying thrombogenic tissue. Although researchers have not reached consensus on the extent of the causative role MPO plays in CVD progression, its correlation is well-established. This has led to research demonstrating that circulating MPO content can predict various clinical outcomes in patients with CVD.

1.2.3 Biomarker utility

Increasing awareness of the role inflammation plays in the initiation, progression, and outcomes of CVD led researchers to focus on inflammation biomarkers to improve preventative and supportive care. Although plasma MPO concentration was first investigated for its biomarker utility in the context of other inflammatory diseases, it was not until the early 21st century that MPO was found beneficial in predicting CVD outcomes. The first report of coronary artery disease (CAD) diagnostic value came in 2001 when the upper quartile of both plasma MPO and intracellular neutrophil MPO predicted CAD status with an odds ratio = 11.9 and 20.4, respectively. Subsequent studies found similar results in a variety of clinical scenarios. MPO predicted both short- and long-term risk of MACEs in patients presenting with ACS. Differentiating ACS from angina pectoris was improved with plasma MPO measurement, as
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was differentiation between stable and unstable angina.\textsuperscript{145–147} Evidence is also growing to support the diagnostic utility of plasma MPO prior to MACE presentation. When measured in patients with diagnosed peripheral artery disease, MPO outperformed C-reactive protein (CRP) in predicting the occurrence of the first MACE, improving stratification already performed using ankle brachial index (ABI).\textsuperscript{148} Other studies found similar correlations between serum MPO and ABI, even after adjusting for serum CRP and other CVD risk factors.\textsuperscript{149,150} Congestive heart failure, which is often a co-morbidity of atherosclerosis, is also associated with plasma MPO.\textsuperscript{151,152} At least one report has shown that MPO can rise early in people with other risk factors for CVD; obese children age 6–12 had plasma MPO correlated with insulin, CRP, and MMPs.\textsuperscript{153} A large study in Germany also showed that MPO in >2,000 randomly selected adults correlates with leisure-time physical activity more than the traditional biomarker oxLDL, suggesting that MPO might even serve as a population-level indicator of public health.\textsuperscript{154} On the opposite end of the CVD spectrum, MPO has also shown utility in assessing post-MI patients.\textsuperscript{155–158} Measurement of the timecourse of rising MPO and the subsequent fall have also demonstrated clinical utility, but this technique may prove difficult given the cost and time requirements of current MPO ELISAs.\textsuperscript{159,160}

The role MPO could serve as a biomarker is still uncertain as other studies have failed to demonstrate increased diagnostic and prognostic value over other indicators. One study found that MPO is only clinically useful when used in conjunction with other biomarkers such as cardiac troponin I, and even then it was outperformed by plasma N-terminal pro-B-type natriuretic peptide in conjunction with troponin I.\textsuperscript{161} Another study in 49 younger adults with acute MI showed no difference in MPO versus controls.\textsuperscript{162} A larger study of 303 patients presenting with ACS and 120 healthy controls found that although MPO was higher in the ACS group, it was not useful in predicting MI or a composite of all-cause mortality with MI,
a finding echoed in an independent study performed soon after.\textsuperscript{163,164} A similarly-powered study in patients with stable CAD found that while MPO predicted mortality during a 5-year study period, it was correlated with other MI risk factors such as age, left-ventricular ejection volume, and poor renal function.\textsuperscript{165}

Although these findings appear to be in direct contradiction with the positive studies, the question of MPO biomarker utility is nuanced. Caveats otherwise ubiquitous in clinical diagnostic studies, such as differences in statistical power, outcome definition, follow-up period, study population, and analysis methods, are applicable to MPO trials. Protocols for measuring MPO are poorly standardized although important confounding factors have been identified. Intravenous heparin and low molecular weight heparins are commonly administered as anticoagulants that function by increasing antithrombin activity.\textsuperscript{166,167} Although heparins have been shown to reduce neutrophil activation and MPO release, it also mobilizes extracellular MPO previously bound to the endothelium resulting in increased measurable plasma concentration.\textsuperscript{137,168–170} Heparin can also affect measured MPO when used as a phlebotomy anticoagulant as demonstrated by studies finding discordant assay results between serum and each of the vacuum tube additives heparin, ethylenediaminetetraacetic acid (EDTA), and citrate.\textsuperscript{171,172} In a recent meta-analysis, these differences in methodology were considered adequate to justify separating studies into protocol-based subgroups for better estimates on MPO utility in the CVD treatment pipeline.\textsuperscript{173} A less-controversial declaration is that more research is warranted to determine the clinical advantages of measuring circulating MPO at various stages of CVD progression. Despite ample numbers of patients along the CVD spectrum (see page 2), current ELISAs and similar methods to measure MPO make such studies a costly proposition. One solution to this problem is to specifically measure
MPO in plasma via an inexpensive and widely-adaptable reporter used commonly outside of medicine: luminescent oxidation of luminol and related compounds.

### 1.2.4 Oxidative bioluminescence

Chemiluminescence is analogous to fluorescence, in which molecular energy of an unstable reaction intermediate is converted into electromagnetic radiation. Unlike with fluorophores excited by incident photons, the energy released in chemiluminescence is derived from potential energy in the bonds of the luminophore. The organic compound 5-amino-2,3-dihydrophthalazine-1,4-dione, better known by the common name luminol, is one of the simplest and most well-studied of the substituted diacylhydrazide class of luminophores. (Figure 1.4A) These compounds release a photon with peak intensity at ~450 nm when the diacylhydrazide moiety is hydrolyzed, releasing molecular nitrogen in the process. (Figure 1.4B) The earliest detailed description of luminol synthesis appeared as part of a graduate thesis defended in 1902. The synthesis was simplified soon after and luminol was studied for the vivid, blue light produced by what was described as “the most striking instance of chemiluminescence.” Its most well-known role today is in the domain of forensics, where its luminescence in the presence of alkaline $\text{H}_2\text{O}_2$ and a metallic catalyst allows crime scene investigators to rapidly identify and photograph trace residues of dried blood.

Bioluminescence is defined as chemiluminescence resulting from biological processes; the phenomenon has arisen through convergent evolution in bacteria, dinoflagellates, fungi, and various animal phyla. Peroxidases, notably HRP, were found to be bioluminescent in the presence of luminol, a property used today to quantify bound immunoglobulin-HRP.

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*Footnote:* Literature searches on the early uses of luminol show many false leads due to the drug phenobarbital, which was discovered and marketed under the name “Luminal” by German pharmaceutical company Bayer AG shortly after luminol was described. Clinical case reports from the first half of the 20th century erroneously mention “luminol” as a sedative or anxiolytic.
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Figure 1.4: Luminol and related luminophores. (A) The structure of chemiluminescent cyclic diacylhydrazides luminol, isoluminol, and L-012. Luminol is a well-studied molecule used in biologic, forensic, and environmental sciences. Isoluminol is similar but comparatively difficult to synthesize; it is used when a probe with decreased cell membrane permeability is required. L-012 is many orders-of-magnitude brighter than either luminol isomer, but is poorly characterized in current literature. (B) The oxidation reaction of luminol and related luminophores which produces chemiluminescence. Deprotonation of the hydrazide drives the reaction in the presence of moderate oxidizing agents. Hypochlorous acid produced by MPO or HRP rapidly oxidizes the luminophore, which is the basis for its use in immunoassays and leukocyte activation studies.

conjugates in protein blots and ELISAs. The ROS produced by HRP are the same as those from the Phox-MPO cascade. As expected, activated leukocytes were soon discovered to be bioluminescent when treated with luminol. Although luminol exhibits chemiluminescence in the presence of a myriad organic and inorganic catalysts, its bioluminescence in neutrophils was soon demonstrated to require the ROS cascade: not only could CGD be detected by the absence of neutrophil bioluminescence, but heterozygous carriers produced ~50% less signal than that from controls. Luminol can also be used as a bioluminescence probe in vivo. Previous work from the D. Piwnica-Worms group has demonstrated luminol specificity for MPO activity both in vivo and in blood ex vivo. Given the ease of synthesis, low cost, and biological specificity for MPO, luminol or similar bioluminescence has the desirable characteristics for a platform upon which a new clinical assay can be developed.
Although luminol has a long history as a luminophore, it is not the most sensitive to oxidation. Other substituted luminophores have been synthesized on the 2,3-dihydrophthalazine-1,4-dione backbone with varying quantum yield. Isoluminol is another diacylhydrazide differing from luminol only in the aromatic-ring position of the primary amine. Although similar in chemiluminescence properties, the two probes differ in their partition coefficients. As meta-substituted isoluminol is more hydrophilic than ortho-substituted luminol, it has been used as a probe for extracellular ROS. A more recently discovered analogue, 8-amino-5-chloro-7-phenyl-2,3-dihydropyrido[3,4-d]pyridazine-1,4-dione (L-012), is far brighter than either luminol isomer when used with cells and cell-free immuno-assays in vitro. (Figure 1.4A) Although the advantage over luminol in terms of detectable photons is clear from early literature reports, publications regarding the specific ROS reported by L-012 are contradictory. Unlike with luminol, imaging studies with L-012 in vivo have not robustly demonstrated the extent of MPO specificity.

The experimental protocols, data, and discussions that follow discuss the investigation of L-012 for the intended purpose of measuring plasma MPO activity in the context of CVD risk stratification, diagnosis, and intervention assessment. To accomplish this, L-012 was first characterized as a bioluminescence probe for MPO activity in vitro and in living tissues with emphasis on extracellular MPO in blood plasma. Conditions for optimum bioluminescence sensitivity and specificity in plasma model systems as well as freshly collected human plasma were determined. Various strategies were then considered to overcome the problems encountered when attempting a direct plasma MPO assay. This resulted in a novel solid-substrate adsorption technique, referred to as the MPO activity on a polymer surface (MAPS) assay, that provided sufficient MPO isolation to produce linear bioluminescence signal for inverse regression against known standards. Lastly, a pilot study with clinical cardiology
patients presenting for elective central catheterization was completed to compare the results of the novel MAPS assay to commercially obtained clinical MPO ELISA kits.

### 1.3 References


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References


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L-012 bioluminescence of MPO in blood

2.1 Introduction

Inflammation plays a key role in a growing list of diseases processes. Its roles in cardiovascular disease (CVD) and the underlying pathology of atherosclerosis have become increasingly central to the understanding of the disease.\(^1\)–\(^6\) Biomolecules in blood plasma indicative of systemic inflammation are the subject of a growing body of research aimed at improving the diagnostic accuracy and detection sensitivity of atherosclerosis progression in pre- and post-symptomatic CVD.\(^7\)–\(^10\) One of the molecules drawing increased interest is the heme-containing antimicrobial enzyme myeloperoxidase (MPO), which is primarily produced in neutrophils and monocytes. MPO is part of the non-specific reactive oxygen species (ROS) cascade initiated by a respiratory burst of \(\text{O}_2\) consumption in neutrophils upon activation by foreign antigen recognition.\(^11\) Although the MPO product hypochlorous acid (HOCl) is important to the microbicidal activity of neutrophils, it can also convert low-density lipoprotein (LDL) into the increasingly pro-inflammatory form oxidized LDL (oxLDL), which is a key driver in differentiating macrophages into the resident foam cells in the cores of necrotic atheromatous plaques.\(^12\)–\(^15\) Neutrophils in the shoulder regions of vulnerable plaques and the re-expression of MPO by macrophages during foam cell differentiation led to studies finding that plasma MPO
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Methods

is predictive of CVD risk, progression, and prognosis.\textsuperscript{16–27} Clinical assays for plasma MPO concentration are enzyme-linked immunosorbent assays (ELISAs) or similar immunochemical methods which are costly, time-intensive, and do not utilize the enzyme activity intrinsic to MPO as a means of readout. The small compound luminol serves as a bioluminescence probe for MPO activity \textit{in vivo} and \textit{in vitro} by emitting a photon upon reaction with HOCl produced by MPO in the presence of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}).\textsuperscript{28} A related compound, L-012, is also oxidized by ROS but produces luminescence orders-of-magnitude greater than that of luminol and has potential to serve as an indicator for plasma MPO activity.\textsuperscript{29,30} Although L-012 has been used to image ROS produced by leukocytes \textit{in vivo}, the specifics of L-012 bioluminescence have yet to be well-studied.\textsuperscript{31,32}

This chapter focuses first on characterizing the chemiluminescence of L-012 in order to investigate questions left unanswered by published studies on the compound. Next, the interaction of L-012 and MPO as a bioluminescence reporter system is investigated for optimization of sensitivity and specificity for ROS produced by MPO activity. Last, attempts to assay MPO in plasma and difficulties caused by plasma components are investigated.

2.2 Methods

2.2.1 Reagents and materials

Lyophilized L-012 was provided by Wako Chemicals USA (Richmond, Virginia; product number 120-04891) was resuspended to 16.1 mM in ultrapure water and stored in 200 µL aliquots at \(-20 ^\circ\text{C}\). Lyophilized human MPO from EMD Millipore/Calbiochem (Darmstadt, Germany; product number 475911) was resuspended in pH = 6.0 buffer containing 100 mM NaCl and 50 mM acetate, as recommended by the supplier, with final concentration 100 mg/L.
Single-use 10 µL aliquots were stored at −20 °C. All other reagents were supplied by Sigma Aldrich (St. Louis, Missouri). Phorbol-12-myristate-13-acetate (PMA) was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. Glucose oxidase (GOx) from Aspergillus niger was resuspended and stored in the same manner as MPO. Modified Earle’s balanced salt solution (MEBSS) was prepared from concentrated stocks to final concentrations 144 mM NaCl, 5.4 mM KCl, 800 µM MgSO₄, 800 µM NaH₂PO₄, 5.6 mM glucose, 4.0 mM HEPES, and 1.2 mM CaCl₂ added last to prevent precipitation. Once mixed, working MEBSS was adjusted with NaOH and HCl to pH = 7.4 and sterilized by filtration. All microtiter plate experiments were performed using 96-well, black-walled, flat-bottom, tissue culture-treated polystyrene microtiter plates (Corning Inc.; Corning, New York; product number 3603).

### 2.2.2 Bioluminescence imaging

Bioluminescence images were obtained with the ONYX/M imaging system with a XR/MEGA-10Z camera (Stanford Photonics; Palo Alto, California), the IVIS100, or the IVIS50 imaging systems (Caliper Life Sciences/PerkinElmer; Waltham, Massachusetts) as noted in the detailed methods descriptions. All whole-blood imaging sequences were obtained with sample plates warmed by a 37 °C stage heater. All IVIS images were obtained as 1 min integrations with F-number = 1, 8×8 pixel binning, and field of view (FOV) = 10 cm unless otherwise noted. IVIS images were exported as raw counts using Living Image 2.60.1 (Xenogen/PerkinElmer). Images in the ONYX/M were obtained with 10 s integrations to prevent camera saturation and combined into 1 min images with the Fiji distribution of ImageJ image analysis software. ONYX data were either gathered as raw luminescence or counts depending on hardware capability, as noted in detailed experiment methods.


2.2.3 Data analysis and statistics

Images were quantified with the Fiji distribution of ImageJ image analysis software. Data from the ONYX/M were processed to correct for optical vignetting as detailed in the Appendix Section §7.4.2 on page 229. Regions of interest (ROIs) were created in microtiter plate arrays using the Quadrangular ROI array program, the source code of which can be found on page 226. Quantifications were normalized to image ROI pixel area to account for inter-experiment differences in ROI layout. All statistical analyses were performed in IBM SPSS Statistics 21 (Armonk, New York) unless otherwise noted. The novel weighted least squares (WLS) regression method is explained in detail in Appendix Section §7.2 and the code for implementing the method can be found on page 212. Nonlinear regression confidence bands were calculated with the delta method.

2.2.4 Human plasma samples

Human blood was obtained from the cephalic vein of healthy volunteers into lithium heparin vacuum tubes for whole blood experiments and K$_2$EDTA tubes for plasma isolation. Plasma was isolated by two-stage centrifugation at 4 °C: 1,000 × g in the collection tubes for 15 min followed by 14,000 × g in microcentrifuge tubes for 10 min. Plasma was stored at −20 °C and thawed at 4 °C before use. The Institutional Review Board at Washington University in St. Louis approved these study protocols.

2.2.5 MPO bioluminescence in blood ex vivo

All solutions were prepared in MEBSS without added serum. Blood was drawn immediately before use. Mouse and rat blood was obtained after anesthetizing the animals with 2% v/v isoflurane in oxygen. Mouse blood was collected by lancet puncture to the facial vein and
allowed to flow into a 400 µL lithium heparinized tube. Rat blood was collected from the lateral tail vein via a 300 µL syringe and a 29.5 gauge needle. Once drawn, the blood was carefully transferred to a 400 µL lithium heparinized tube. Data in Figure 2.1A were obtained by mixing 50 µL blood with 50 µL PMA or DMSO pre-mixed with luminol, final concentration 100 µM, and imaging in the ONYX/M. Data in Figure 2.2 and Figure 2.3 were obtained by combining 100 µL mixed blood and bioluminescent probe with 100 µL PMA or DMSO in a microtiter plate; final concentrations for each experiment were 0.5% v/v blood, 100 µM luminol or L-012, and 5 µM PMA or equivalent 0.5% v/v DMSO and imaged in the IVIS100. Data in Figure 2.4 were obtained by combining 25 µL PMA or DMSO, 25 µL 4-aminobenzoic hydrazide (4-ABH) dilution, and 50 µL diluted rat blood before imaging in the IVIS100 with 16×16 binning and a 15 cm FOV.

2.2.6 MPO halogenation kinetics

Halide bioluminescence data shown in Figure 2.5A were obtained by making a halide-free phosphate buffer (5.19 mM NaH$_2$PO$_4$ and 4.81 mM Na$_2$HPO$_4$, pH = 7.0) and supplementing with sodium halide salts. Bioluminescence was initiated by arraying a microtiter plate with 50 µL H$_2$O$_2$ (200 µM final concentration) and adding 150 µL of a halide solution containing L-012 (final concentration 100 µM) ±MPO in the same phosphate buffer. Individual halide titrations shown in Figure 2.5B were performed in an acidic citrate buffer system (3.09 mM citric acid and 21.9 mM trisodium citrate, calculated pH ($^{C\text{pH}}$) = 6.0). 45 µL buffered H$_2$O$_2$ with a final concentration 100 µM was added to 35 µL (pseudo)halide solution in each well. The reaction was initiated by adding 150 µL L-012 (final concentration 35 µM) ±MPO.

Cross-titrations of Cl$^-$ and Br$^-$ shown in Figure 2.6 were performed by serially diluting NaCl and NaBr separately in water before arraying 50 µL of each halide solution in quadruplicate
into microtiter plate wells. Each of the 100 µL mixed halide solutions in the wells received 50 µL solutions prepared to give final concentrations of 8.84 mM NaH$_2$PO$_4$, 1.22 mM Na$_2$HPO$_4$, 100 µM L-012, and either 500 ng/L MPO diluted into Dulbecco’s phosphate buffered saline (DPBS) or buffer control. The reaction was initiated by the addition of H$_2$O$_2$ with a final concentration of 200 µM before immediate photon counting in the ONYX/M.

### 2.2.7 MPO bioluminescence with glucose oxidase H$_2$O$_2$ generation

Cross titrations in Figure 2.7 were performed in DPBS-buffered solutions mixed in a 96-well microtiter plate and imaged in the ONYX/M with photon-counting enabled. Glucose and GOx cross-titration reactions in Figure 2.7A were initiated by sequentially mixing 50 µL L-012 (15 µM final concentration), 50 µL GOx, and 100 µL glucose. MPO and GOx cross-titration reactions shown in Figure 2.7B were initiated by sequentially mixing 50 µL MPO, 50 µL GOx, and 100 µL pre-mixed L-012 and glucose (final concentrations 30 µM and 20 mM, respectively).

### 2.2.8 Bioluminescence spanning linear pH titrations

In order to increase precision in measuring the effects of pH on MPO-dependent and -independent oxidation of luminol and L-012, buffers with overlapping pK$_a$ coverage were tested with an electronic pH meter. Buffer planning and simulated titrations were completed in CurTiPot v3.6.1. The buffer system used in Figure 2.8 was chosen for its wide, near-linear buffering range. The final solution contained 2.4 mM of each: Na$_2$EDTA dihydrate, Tris HCl, lysine, and trisodium citrate, the pK$_a$s of which are shown in Figure 2.8A. Buffer capacity was measured at room temperature with stepwise addition of ~5 M NaOH or ~12 M HCl,
shown compared to the CurTiPot predictions in Figure 2.8B. Stock buffer solution titrations to pH = 4.0, 5.0, 6.0, 7.0, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0 were made with the same HCl and NaOH, filter sterilized, and stored at 4 °C for future use. Basal rate chemiluminescence shown in Figure 2.8C was measured by serially diluting H2O2 at into each of the buffer solutions, arraying two 96-well microtiter plates (in groups of 11 as they were imaged) with 5 µL of either luminol or L-012 in water. Each reaction was initiated by adding 95 µL each pH×H2O2 solution to a plate row containing one of the two probes every 45 s. The signal was strongly dependent on reaction duration, so each 30 s image was taken 45 seconds apart in the IVIS100 (8×8 binning, 15 cm FOV) so each row had an image taken at the same reaction timepoint.

A simplified, mostly inorganic buffer system was chosen for pH experiments involving MPO in order to minimize the chance of pharmacological effects from the buffer components. The buffer was prepared with 120 mM NaCl and 10 mM of each NaH2PO4, B(OH)3, and sodium acetate. The pH was adjusted with ~5 M NaOH as shown compared to the CurtTiPot prediction in Figure 2.9A. Large stock volumes of buffered pH = 6.0, 6.5, 7.0, 7.4, 8.0, and 9.0 were filter-sterilized and stored at 4 °C until use. MPO solutions were prepared in corresponding buffers accounting for dilution to give 50 µg/L final MPO concentration. Solutions of final concentrations 0, 10, 100, and 1,000 µM H2O2 in water were prepared accounting for dilution into the reaction volume, as were solutions of 10 µM either luminol or L-012 in water. Reactions were prepared by sequentially aspirating 85 µL of a specific pH buffer, 5 µL air, 5 µL of the luminol or L-012 solution, 5 µL air, 5 µL H2O2 solution, and a final 5 µL of air into tips of a multichannel pipette. Reactions were initiated by rapidly expelling the entire tip volume into microtiter plate wells, the large bolus of buffer serving as a turbulent mixing step. Imaging began precisely 3 s after addition with the normal
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maximum instrument gain = 1.750 for luminol plates and gain = 1.400 for L-012 solutions to prevent camera saturation or damage. Ratios from the 10 µM H₂O₂ series taken at the 2 min timepoint are shown in Figure 2.9B and were calculated using log-linked gamma regression.

Solubility curves for L-012 in acidic buffer were measured by serially diluting L-012 into water before adding to pH = 5.75 buffer containing 4.50 mM citric acid, 20.5 mM trisodium citrate, and 250 mM NaBr in 1 mL polystyrene cuvettes. Absorbance at 550 nm was measured every 5 s for 2 min and the slope from unweighted linear regression was calculated with 95% confidence intervals.

2.2.9 Plasma filtrate titration

Plasma inhibition titrations shown in Figure 2.11 were obtained by first preparing centrifugal ultrafiltration concentrators (Corning Life Sciences Spin-X tubes with 5 kDa and 100 kDa filtration membranes, product numbers 431482 and 431486) by filtering 6 mL ultrapure water for 20 min at 4 °C, 4,000 × g to remove contaminants from the manufacturing process. Plasma was collected from the cephalic vein of a healthy volunteer into a K₂EDTA vacuum tube as described on page 42. Plasma was diluted to 0.5% v/v in pH = 6 citrate buffer containing 3.1 mM citric acid, 22 mM trisodium citrate, and 10 mM NaBr, which was then loaded into the filtration tubes and spun for 1 h at 4 °C, 4,000 × g. Control plasma was diluted into the same buffer and stored at 4 °C for the centrifugation duration to serve as a control. Filtrates or dilute whole plasma were further diluted into the same buffer along with reagents adjusted for final reaction volumes to give 500 ng/L MPO and 50 µM L-012. Reactions were initiated by arraying microtiter plates with 10 µL H₂O₂ (final concentration 50 µM) and adding 75 µL diluted plasma filtrates supplemented with MPO and L-012 before immediately imaging raw luminescence in the ONYX/M. Bioluminescence was intensity was
integrated for 1 min and fit to the equation

\[ B = \frac{B_{\text{all max}}IC_{50}^H}{[\text{filtrate}]^H + IC_{50}^H} \]  

(2.1)

to quantify the extent of plasma inhibition. The term \( B_{\text{all max}} \) refers to the common asymptotic maximum shared by all plasma filtrates, while the terms \( H \) and \( IC_{50} \) were calculated for each filtrate. Confidence bands were calculated using the delta method.\(^{34}\)

### 2.2.10 Antioxidant titration

Titration curves to quantify bioluminescence inhibition by ascorbic and uric acids shown in Figure 2.12 were obtained with similar experimental protocols. A final in-well MPO concentration of 80 ng/L was chosen based on 20 µg/L MPO, typical of a healthy donor, at a sample dilution of 0.4% \( v/v \) based on previous plasma bioluminescence assays. Both series of experiments were performed in \( \text{pH} = 6.0 \) citrate buffer containing 20 mM NaBr, 3.05 mM citric acid, and 22.0 mM trisodium citrate. The ascorbic acid titration shown in Figure 2.12A was obtained by co-diluting ascorbic acid with constant concentrations of MPO (80 ng/L final concentration) and L-012 (30 µM final concentration). The oxidizer solution contained buffered \( \text{H}_2\text{O}_2 \) at a final concentration 100 µM. The uric acid titration in Figure 2.12B also included 200 ppm \( v/v \) polysorbate 20 (Tween20) in the dilution buffer to reduce inter-replicate variance. 30 µM final L-012 was added to the oxidizer solution along with \( \text{H}_2\text{O}_2 \) immediately before imaging. In both experiment series, 75 µL of each antioxidant solution was arrayed into a microtiter plate in quadruplicate and imaging raw luminescence was initiated in the ONYX/M immediately after the addition of 75 µL oxidizer solution. Inhibition data were quantified from 1 min exposures taken at the 1 min timepoint. Effects
of ascorbic acid were quantified using equation

\[ B = B_{\text{min}} + \frac{(B_{\text{max}} - B_{\text{min}}) IC_{50}^H}{IC_{50}^H + [\text{ascorbic acid}]^H} \] (2.2)

where \( B_{\text{min}} \) and \( B_{\text{max}} \) are the asymptotic minimum and maximum, \( H \) is the Hill coefficient, and \( IC_{50} \) is the concentration of ascorbic acid with half-maximal inhibition. Uric acid inhibition was regressed with the same equation, but the \( F \)-ratio test determined the term \( B_{\text{min}} \) to be insignificantly different from 0, thus the simplified equation

\[ B = \frac{B_{\text{max}} IC_{50}^H}{IC_{50}^H + [\text{uric acid}]^H} \] (2.3)

required fewer degrees of freedom and thus more precision in the remaining terms \( H \) and \( IC_{50} \). Confidence bands were calculated using the delta method.\(^{34}\)

### 2.3 Results

#### 2.3.1 L-012 ROS sensitivity compared to luminol in whole blood

*ex vivo*

Previously published data demonstrate that luminol is specific for MPO activity *in vivo* and in blood *ex vivo*, but the luminol analogue L-012 is not as well-studied and reports on the ROS detected by L-012 differ across the literature.\(^{28-30,36-38}\) Although luminol was sensitive enough to detect ROS production in blood diluted below 0.1% \( v/v \), L-012 had sufficient sensitivity to detect single-cell bioluminescence. (Figure 2.1)

To directly compare the sensitivity of luminol and L-012, fresh blood collected from adult male Sprague-Dawley rats and C57BL/6 mice were diluted into MEBSS with each probe. Rats have more circulating neutrophils than mice, and of the common inbred mouse strains,
Figure 2.1: Bioluminescence of human blood \textit{ex vivo}. (A) Serial dilutions of whole human blood from a healthy volunteer into MEBSS and imaged with 100 µM luminol after treatment with 5 µM PMA or 0.5% \textit{v/v} DMSO control. Raw data from individual replicates are shown ($n = 4$). (B) Images of individual blood cells treated with 1 µM PMA and 50 µM L-012 in an MEBSS imaging solution. The skin of a healthy volunteer was pricked with a 29.5 gauge needle, which was then gently agitated into imaging solution. These images were obtained by integrating luminescence for 1 min at the 10 min timepoint.
C57BL/6 mice have relatively limited acute inflammation activity;\textsuperscript{39-42} as expected from this, rat blood produced more bioluminescence signal with either probe when treated with PMA. Blood from either animal had similar times to peak bioluminescence of 15–20 min, but rat blood required more time for signal to decay back to the DMSO-treated control baseline. L-012 was \~20–40-fold brighter than luminol in blood from both animals. (Figure 2.2A) Sensitivity (signal ratio of PMA-induced bioluminescence to DMSO-treated control) was also greater with L-012 by a factor of \~2. (Figure 2.2B)

### 2.3.2 L-012 specificity for whole-blood MPO activity

To directly compare the specificity of luminol and L-012 for MPO activity, bioluminescence with each probe using fresh blood from \textit{Mpo}^{-/-} and \textit{Mpo}^{+/-} C57BL/6 mice was compared. Bioluminescence from the \textit{Mpo}^{+/-} mice was as previously observed. (Figure 2.3A) Luminol bioluminescence did not differentiate \textit{Mpo}^{-/-} blood treated with PMA from that treated with DMSO. \textit{Mpo}^{-/-} blood imaged with L-012 after PMA treatment showed increased bioluminescence compared to DMSO control but rapidly reached a sharp threshold to a signal plateau that was maintained for the duration of the time series. (Figure 2.3B)

Specificity of L-012 for MPO activity was also verified pharmacologically with the MPO inhibitor 4-ABH. L-012 bioluminescence in whole blood from Sprague-Dawley rats treated with PMA showed similar initial rates of increase in the first 5 min of imaging. Peak bioluminescence output was inversely proportional to 4-ABH concentration and was reached earlier with more inhibitor present. (Figure 2.4A) Regression using bioluminescence from each peak timepoint shows that 4-ABH inhibits L-012 bioluminescence with an \textit{IC}_{50} = 63 \mu M (95\% confidence interval 48–78 \mu M), consistent with literature reports of MPO inhibition.\textsuperscript{28,43} (Figure 2.4B)
Figure 2.2: L-012 bioluminescence in rat and mouse blood \textit{ex vivo}. (A) 0.5% \textit{v/v} blood from either C57BL/6 mice or Sprague Dawley rats in MEBSS imaged with 100 µM luminol or L-012 after treatment with 5 µM PMA or 0.5% \textit{v/v} DMSO control. (B) Signal from PMA-treated blood normalized to DMSO control by log-linked gamma regression. Colored bands are 95% confidence intervals about the mean ratio ($n = 4$). Dashed line denotes a ratio $= 1$. 

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Figure 2.3: ROS specificity of L-012 in Mpo<sup>−/−</sup> blood ex vivo. (A) 0.5% <i>v/v</i> blood from Mpo<sup>+/+</sup> or Mpo<sup>−/−</sup> C57BL/6 mice imaged with 100 µM luminol or L-012 after treatment with 5 µM PMA or 0.5% <i>v/v</i> DMSO control. Raw data (<i>n</i> = 4) are shown. (B) Bioluminescence from PMA-treated blood normalized to signal from DMSO-treated controls. Ratios were determined by log-linked gamma regression and are shown as mean ± 95% confidence intervals. Dashed line denotes a ratio = 1.
2.3.3 Halide substrates for MPO bioluminescence

Literature reports of MPO activity in the presence of various (pseudo)halide electron donors suggest that physiologic Cl\(^-\) is the least effective in reducing MPO compound-I back to native MPO: Br\(^-\), I\(^-\), and SCN\(^-\) have incrementally higher affinity.\(^{44-47}\) From this, it was expected that bioluminescence intensity would have the same relationship to halogenation substrate. Preliminary data confirmed that MPO-dependent bioluminescence in the presence of Br\(^-\) was many orders-of-magnitude brighter than that using only Cl\(^-\), but I\(^-\) failed to differentiate MPO from buffer. (Figure 2.5A) Titration of each of the four substrates provided stronger evidence that L-012 bioluminescence is not indicative of all MPO activity but rather of chlorination and, more so, bromination to respectively generate HOCl and HOBr. Bioluminescence substrate efficiency was quantified by approximating the complex kinetics of MPO (see Figure 1.3 on page 13) with the Michaelis-Menten equation, showing a \(K_m = 17\) mM (95% confidence
Luminescence / pixel–1 min–1

<table>
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Figure 2.5: MPO halogenation substrate kinetics. (A) Bioluminescence time series of MPO halogenation in the presence of 200 µM H₂O₂, 100 µM L-012, and sodium halide salts in pH = 7.0 phosphate buffer. Insets in the NaCl and NaI plots are the same data vertically scaled according to the axis label to show detail. 150 mM NaCl allowed some MPO resolution, but this was quenched at 500 mM. Despite literature reports of I⁻ as the most reactive halide for MPO halogenation, NaI did not differentiate MPO from buffer. Of the three true halides, Br⁻ bioluminescence was orders-of-magnitude brighter. (B) Separate titration curves of (pseudo)halides ± MPO with 100 µM H₂O₂ and 30 µM L-012 in a C pH = 6.0 citrate buffer. Bioluminescence was measured for 1 min after mixing reagents in a microtiter plate. Both Cl⁻ and Br⁻ can be fit as Michaelis-Menten substrates: Cl⁻ giving a \( K_m = 17 \) mM (95% confidence interval 10–23 mM) and Br⁻ giving \( K_m = 2.3 \) mM (95% confidence interval 1.7–2.9 mM). Data are plotted as mean ± standard deviation (\( n = 4 \)). Regression with shaded 95% confidence bands are shown where applicable.

interval 10–23 mM) for Cl⁻ and \( K_m = 2.3 \) mM (95% confidence interval 1.7–2.9 mM) for Br⁻. The \( \sim 10 \)-fold difference in \( K_m \) as well as bioluminescence signal magnitude are consistent with the literature. However, MPO reactions involving I⁻ and SCN⁻ did not allow for similar bioluminescence, even over a wide range of substrate concentrations. (Figure 2.5B) Bromination is inhibited by excess Cl⁻, as expected if Cl⁻ is a competing MPO substrate with slower kinetics. In the absence of Br⁻, chlorination bioluminescence is saturable with excess
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Figure 2.6: Chloride and bromide as competing MPO substrates. Cross-titration of Cl\(^-\) and Br\(^-\) in the presence of 100 µM L-012, 200 µM H\(_2\)O\(_2\), and 500 ng/L MPO or buffer control. Raw bioluminescence data (n = 4) from the 5 min timepoint are shown. (A) Bioluminescence increases with Cl\(^-\) in the absence of Br\(^-\). In the presence of Br\(^-\), Cl\(^-\) acts as an inhibitor. (B) The same data as in (A) arranged to show the effect of Br\(^-\). MPO bioluminescence is saturable with Br\(^-\) at all concentrations of Cl\(^-\). Peak bioluminescence occurs with Br\(^-\) in the absence of Cl\(^-\).

Cl\(^-\), but it decreases signal when Br\(^-\) is available. (Figure 2.6A) The same data show the saturable kinetics of bromination with a lower asymptote with increasing Cl\(^-\). (Figure 2.6B)

2.3.4 Hydrogen peroxide effects on MPO kinetics

H\(_2\)O\(_2\) has the potential to function as both a substrate for MPO bioluminescence via halogenation cycling and an inhibitor via shunting MPO mass into the slower peroxidation cycles. (Figure 1.3) In order to investigate the effects of H\(_2\)O\(_2\) concentration on MPO bioluminescence with L-012, H\(_2\)O\(_2\) was generated with GOx and substrate d-glucose. This allows reaction duration to be used as an approximation for H\(_2\)O\(_2\) concentration as an
alternative to discrete concentrations varying between solutions. The assumption of linear 
\( \text{H}_2\text{O}_2 \) generation by this system over time was tested with direct L-012 oxidation. Linear 
increase in signal across a wide range of GOx concentrations in the presence of 20 mM 
\( \text{D}-\text{glucose} \) were obtained for >1 h. (Figure 2.7A) Using this system, the inhibitory effect 
of excess \( \text{H}_2\text{O}_2 \) on MPO bioluminescence was clearly observable. Direct L-012 oxidation 
by \( \text{H}_2\text{O}_2 \) occurred in the absence of MPO as previously observed, but in the presence of 
MPO bioluminescence fell after a duration made shorter by increasing concentrations of GOx. 
(Figure 2.7B) This confirmed that excess \( \text{H}_2\text{O}_2 \) will inhibit MPO bioluminescence as suggested 
by the literature, but this \( \text{H}_2\text{O}_2 \) effect on MPO activity is known to be pH-dependent.\textsuperscript{49,50}

### 2.3.5 Optimizing pH for MPO detection with L-012

In order to investigate baseline oxidation of L-012 in a wide range of pHs, a linear buffer with 
dispersed \( pK_a \)s was designed (Figure 2.8A) and tested against the predicted titration curve 
from pH = 4 to pH = 11. (Figure 2.8B) As hypothesized, luminol was oxidized more readily 
with increasing pH but was not significantly affected until pH > 7. L-012 was similarly more 
sensitive to direct oxidation by \( \text{H}_2\text{O}_2 \) at higher pH, but began to be susceptible at pH > 5. 
Unexpectedly, background chemiluminescence from both probes depended more on pH than 
\( \text{H}_2\text{O}_2 \) concentration, suggesting that non-specific signal can be eliminated by optimizing 
solution conditions. (Figure 2.8B)

A simplified buffer system with a near-linear titration from pH = 6 to pH = 11 was 
designed to test the combined effects of pH on MPO activity and resulting luminol and L-012 
oxidation. This system contained fewer components to reduce the risk unforeseen buffer 
interactions, did not contain chelators that could potentially disrupt the heme cofactors of 
MPO, and was free of amines that could react with MPO-produced HOCl. As with the
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Figure 2.7: Generating $\text{H}_2\text{O}_2$ with GOx. Using glucose and GOx instead of discrete $\text{H}_2\text{O}_2$ solutions allowed for precise observation of MPO bioluminescence kinetics by using reaction duration as an approximation of relative $\text{H}_2\text{O}_2$ concentration. (A) Bioluminescence time series of cross-titrated d-glucose and GOx with 15 µM L-012 in DPBS. The ONYX/M detected non-specific L-012 oxidation by $\text{H}_2\text{O}_2$ produced by the glucose-GOx system. Signal intensity was proportional to substrate glucose concentration through multiple orders of magnitude, but it inhibited bioluminescence at high concentrations. Moderate glucose concentrations produced linear $\text{H}_2\text{O}_2$ for >1 h. (B) Bioluminescence time series of GOx titrated ±MPO in the presence of 30 µM L-012 and 20 mM d-glucose. MPO-dependend bioluminescence was detectable above direct L-012 oxidation by $\text{H}_2\text{O}_2$. Intensity and time of the transient signal peaks were influenced by GOx concentration. At higher concentrations, $\text{H}_2\text{O}_2$ shifted from an MPO bioluminescence substrate to an inhibitor. This observation agrees with reported electrochemical observations of MPO kinetics in the literature.\(^{49}\)
Figure 2.8: Luminol and L-012 lability varies with pH. (A) Buffer components with dispersed pKₐs allowing for a wide, near-linear pH titration. (B) Titration curve of ~5 M NaOH and ~12 M HCl into 50 mL buffer containing 2.4 mM each of EDTA, Tris, citrate, and lysine. OH⁻ equivalents are defined as \( \sum \text{NaOH} - \sum \text{HCl} \). Colored bars along pH axis correspond to pKₐ values for buffer components shown in part (A). Dashed line shows simulated titration values obtained with CurTiPot v3.6.1.³⁵ (C) Linear and semi-log plots of 30 s images of the four-component buffer with 100 µM luminol or L-012 and varying H₂O₂. Each reaction was imaged 15 s after H₂O₂ was added to the bioluminescent probe. Raw data (n = 4) are shown.
previous buffer system, observed titration closely matched the simulations performed in CurTiPot. (Figure 2.9A) Gamma regression to determine the signal ratio of 50 µg/L MPO to buffer control showed that luminol was less susceptible than L-012 to the effects of pH on MPO detection. Although L-012 was consistently brighter than luminol, its sensitivity is poor above pH = 7 due to direct oxidation by H₂O₂. However, its sensitivity was ~100-fold greater than that of luminol at pH = 6. (Figure 2.9B) Observations of L-012 precipitation in buffered stock solution tubes raised concerns about solubility at low pH; aggregation on pipette- or assay surfaces is a potential source of experimental error. L-012 began to precipitate at concentrations > 60 µM in a pH = 5.75 buffer, suggesting a compromise between pH requirements for MPO specificity and L-012 solubility should be used to assay MPO activity. (Figure 2.9C)

Literature reports that MPO kinetics shift to favor halogenation over peroxidation in acidic solutions, allowing for higher concentrations of substrate H₂O₂ without loss of MPO activity.⁴⁹,⁵⁰ Pure human MPO imaged with L-012 in an acetate buffer with a pH₁ = 5.0 resulted in exponential decay of bioluminescence over time as expected if H₂O₂ was not shifting MPO away from the halogenation reaction. (Figure 2.10A) Bioluminescence was linear with MPO concentration up to 1 mM H₂O₂, and regression slopes increased with H₂O₂ as well. (Figure 2.10B)

The improvements seen with low-pH imaging solutions did not translate to MPO in human plasma, however. MPO-independent bioluminescence were not a major confounding variable with H₂O₂ concentrations below ~250 µM; reduced overall signal in acidic buffer compounded the loss of signal associated with imaging MPO in the presence of plasma proteins, making MPO measurement imprecise. (Figure 2.10C) Even after normalizing to baseline signal, imaging with L-012 in mildly acidic buffer showed improved MPO resolution
Figure 2.9: pH-dependent MPO resolution with luminol and L-012. (A) Titration curve of ~5 M NaOH into 45 mL of a near-linear buffer system containing 10 mM PO$_4^{3-}$, Ac$^-$, and B(OH)$_3$. Each $pK_a$ and conjugate base relevant to the pH range is annotated along the pH axis. Dashed line shows simulated titration values obtained with CurTiPot v3.6.1. $^{35}$  (B) Ratio of bioluminescence intensity (50 µg/L MPO:buffer) in the presence of 10 µM H$_2$O$_2$ and 10 µM luminol or L-012 integrated for 1 min after a 2 min incubation in the ONYX/M. Ratios were calculated using log-linked gamma regression and are plotted as mean ± 95% confidence interval. (C) Precipitation rates of L-012 in buffer made from 250 mM NaBr, 20.5 mM trisodium citrate and 4.50 mM citric acid, with pH = 5.75. L-012 was mixed with buffer in a 1 mL plastic cuvette and continuously monitored for changes in 550 nm absorbance in a spectrophotometer over 2 min. Changes in absorbance were linear for this duration, due to pre-equilibrium precipitation of L-012 onto the cuvette wall. Linear regression slope parameter ($\Delta$Abs$_{550}$ / min) ± 95% confidence interval are shown.

despite the use of strongly acidic buffer across an order-of-magnitude range of H$_2$O$_2$ concentrations. (Figure 2.10D)

2.3.6 MPO inhibition by plasma

Titrating fresh plasma from a healthy donor directly into an acidic buffer containing MPO, L-012, and H$_2$O$_2$ resulted in an $IC_{50} = 28$ ppm $v/v$ (95% confidence interval 21–35 ppm $v/v$) and a Hill coefficient $H = 0.59$ (95% confidence interval 0.51–0.67) when regressed with equation (2.1). Ultrafiltration to selectively remove plasma components resulted in more abrupt inhibition across the dilution range with $H = 1.2$ (95% confidence
Figure 2.10: Acidic L-012 MPO resolution with varying \( \text{H}_2\text{O}_2 \). (A) Time series of MPO bioluminescence with 40 µM L-012 diluted into buffer containing 1 mM EDTA, 150 mM NaBr, 16.7 mM sodium acetate, and 8.33 mM acetic acid with a \( \text{pH} = 5.0 \). Raw data \((n = 4)\) from 1 min exposures in the ONYX/M are shown. (B) MPO resolution from these data taken at the 5 min timepoint. Data are plotted as mean ± standard deviation. WLS regression ± 95% confidence bands are shown as dashed lines and shaded areas, respectively. (C) Time series of MPO bioluminescence with human plasma ± 100 µg/L MPO diluted to 0.1% \( v/v \) in buffer containing 1 mM EDTA, 20 mM NaBr, 10 µM L-012, and varying \( \text{H}_2\text{O}_2 \). Each of the pH solutions contains 25 mM of their respective buffering agent. Raw bioluminescence data \((n = 4)\) are shown. MPO is annotated with pre-dilution concentrations. (D) Signal ratio at the 5 min timepoint of the time series shown in (C). Ratios are presented as mean ± 95% confidence interval as determined by log-linked gamma regression. Dashed line indicates a ratio = 1.
Figure 2.11: Whole plasma inhibition of MPO. (A) Titration curves of plasma filtrates in the presence of 500 ng/L MPO, 50 µM L-012, and 10 mM NaBr buffered with 3.05 mM citric acid and 21.9 mM trisodium citrate. Bioluminescence was measured for 1 min after adding H₂O₂ with a final concentration of 100 µM. MPO inhibition by plasma was quantified by equation (2.1). Whole plasma inhibited MPO bioluminescence with an IC₅₀ = 28 ppm v/v (95% confidence interval 21–35 ppm v/v) and H = 0.59 (95% confidence interval 0.51–0.67). Filtration shifted the values of the IC₅₀ to 120 ppm v/v (95% confidence interval 110–140 ppm v/v) and the Hill coefficient H to 1.2 (95% confidence interval 1.1–1.3), resulting in sharper and right-shifted inhibition curves. Data are shown as mean ± standard deviation (n = 4). Nonlinear regression and 95% confidence bands are shown as dashed lines and shaded areas, respectively. (B) Regressed values of IC₅₀ for each plasma filtrate shown in part (A). Filtration cutoffs 5 and 100 kDa were not significantly different by IC₅₀ or H (p = 0.21 and 0.30, respectively). Data are presented as the estimate ± 95% confidence interval.

Ultrafiltration also increased the IC₅₀ to 120 ppm v/v (95% confidence interval 110–140 ppm v/v). (Figure 2.11A) There was no significant difference between 5 kDa and 100 kDa ultrafiltration by either IC₅₀ (p = 0.21) or H (p = 0.30). (Figure 2.11B)

To determine the extent of small-molecule antioxidants to MPO bioluminescence inhibition by plasma, two major physiological antioxidants were titrated with pure MPO in the presence of H₂O₂ and L-012. An MPO concentration of 80 ng/L was chosen to represent a 0.4% v/v dilution of 20 µg/L MPO plasma. Ascorbic acid was the more potent antioxidant with an IC₅₀ = 36 nM (95% confidence interval 31–41 nM) as determined by equation (2.2). As ascorbic acid concentration approached the dilution-adjusted physiologic reference range of 140–360 nM, MPO-dependent bioluminescence was almost entirely quenched. (Figure 2.12A)
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Figure 2.12: Antioxidant contribution to MPO inhibition. Physiological small-molecule antioxidants (A) ascorbic acid and (B) uric acid titrated in the presence of 20 mM NaBr, 200 ppm v/v Tween20, 80 ng/L MPO, and 30 μM L-012 in buffer containing 3.05 mM citric acid and 22.0 mM trisodium citrate. MPO concentration was chosen to recapitulate plasma with a physiologic level of 20 μg/L MPO diluted to 0.4% v/v. Ascorbic acid titration data were regressed using equation (2.2) and uric acid data with equation (2.3). Physiologic reference ranges adjusted for the 0.4% v/v dilution are shown as black bars. Ascorbic acid inhibited MPO bioluminescence with an IC₅₀ = 36 nM (95% confidence interval 31–41 nM) and signal was quenched to baseline at the dilution-adjusted reference range 140–360 nM. Uric acid inhibited MPO bioluminescence with an IC₅₀ = 150 nM (95% confidence interval 120–180 nM), within the dilution-adjusted reference ranges for males (96–220 nM) and females (64–170 nM). Data are shown as mean ± standard deviation (n = 4). Nonlinear regression results are shown as dashed lines ± 95% confidence bands in gray. Physiologic reference ranges from McPherson & Pincus.51

Uric acid also potently inhibited MPO bioluminescence but with a higher IC₅₀ = 150 nM (95% confidence interval 120–180 nM) by equation (2.3). This is similar to the dilution-adjusted reference range for adults of either sex, (96–220 nM) and (64–170 nM) for males and females, respectively.51 (Figure 2.12B)

To determine if antioxidant inhibition could be mitigated by consumption with H₂O₂, the d-glucose/GOx system was used in the presence of varying dilutions of human plasma. Unlike as previously seen in buffer (see Figure 2.7A), bioluminescence showed biphasic kinetics as H₂O₂ accumulated. The timing and duration of the transition from low- to high-slope kinetics depended on plasma dilution more so than on the presence of exogenous MPO. (Figure 2.13A) Despite this, each plasma dilution shared the timepoint of maximum MPO resolution. Beyond
~10 min, MPO-independent L-012 oxidation resulted in converging bioluminescence from plasma ± MPO. (Figure 2.13B) At the 10 min timepoint, unsupplemented plasma dilution showed a linear relationship with bioluminescence inhibition. In contrast, plasma with exogenous MPO did not show a linear relationship between dilution and bioluminescence signal. (Figure 2.13C)

2.4 Discussion

Inflammation is well-established as a central driver of the atherosclerosis responsible for the majority of CVD morbidity and mortality. Detection and quantification of acute inflammatory cells, primarily neutrophils, with the chemiluminescent compound luminol has been demonstrated in the literature. Luminol bioluminescence is MPO-dependent in vivo and in blood ex vivo; plasma MPO content is also a rising biomarker for CVD risk and progression. The luminol derivative L-012 has more sensitivity to oxidation than luminol, but little is known about its specificity for ROS and its behavior when used as a bioluminescence reporter in vivo and in vitro. Here, L-012 is characterized for use as a bioluminescent reporter of MPO activity in the context of application to a plasma MPO assay.

Although luminol can detect neutrophil activation in human blood diluted to <0.1% v/v, L-012 can be used to visualize single cells undergoing a respiratory burst. (Figure 2.1) The increased oxidation sensitivity of L-012 is apparent when compared directly to luminol with whole blood imaging, as in Figure 2.2: total luminescence is increased by a factor of ~40 and sensitivity to leukocyte activation increased by a factor of ~2. Despite the clear improvement in oxidation detection, surprisingly little has been published on L-012 chemistry and reports on
Figure 2.13: Nonlinear plasma inhibition of MPO bioluminescence. (A) Time series of plasma ± 150 µg/L MPO serially diluted in the presence of 1 mM EDTA, 50 µg/L GOx, 200 mM D-glucose, 130 mM NaBr, and 30 µM L-012 buffered with 35 mM Na$_2$HPO$_4$ and 15 mM NaH$_2$PO$_4$ (pH = 7.2). Lines represent raw signal of individual wells (n = 4). (B) Signal ratio of plasma + MPO normalized to untreated plasma at each dilution. Mean ± 95% confidence band as determined by log-linked gamma regression are shown for each dilution across the time series. (C) Raw data from the image captured 10 min into the H$_2$O$_2$ production by GOx. Oxidation of L-012 is inhibited by untreated plasma with a strong linear trend. Bioluminescence from plasma supplemented with MPO increases with total concentration until 400–500 ppm v/v, above which the inhibition from plasma components overcomes the increasing MPO activity.
its sensitivity and specificity for different ROS are often contradictory.\textsuperscript{29,30,36–38} As previously shown with luminol, L-012 has specificity for MPO activity \textit{in vivo}, although there is some MPO-independent background luminescence from PMA-activated blood from \textit{Mpo}\textsuperscript{−/−} mice.\textsuperscript{28} (Figure 2.3) The kinetics of \textit{Mpo}\textsuperscript{−/−} blood bioluminescence and the relatively weak PMA response characteristic of C57BL/6 mice suggests that the nearly absolute specificity of luminol for MPO activity in blood \textit{ex vivo} may be a result of instrumentation and poor quantum yield as opposed to intrinsic luminol chemistry. MPO-dependent bioluminescence composes an overwhelming majority of overall detectable signal from more robust acute inflammatory systems, as evidenced by the effect of the potent, specific MPO inhibitor 4-ABH on PMA-activated rat blood. (Figure 2.4)

Although L-012 bioluminescence reports MPO activity from activated leukocytes in diluted blood, the optimal reaction conditions, substrates, and product ROS for measuring MPO activity are not known. Of the four (pseudo)halide substrates for MPO compound I, SCN\textsuperscript{−} and I\textsuperscript{−} have the greatest rate constants and that of Br\textsuperscript{−} is only slightly lower; the physiological substrate Cl\textsuperscript{−} is many orders of magnitude slower to react with MPO.\textsuperscript{46} This suggests that NaBr is a more suitable MPO assay reagent than NaCl, which was verified experimentally. As expected, Cl\textsuperscript{−} and Br\textsuperscript{−} were competitive substrates for MPO halogenation and the resulting L-012 bioluminescence. (Figure 2.6) Contrary to the literature-based hypothesis, SCN\textsuperscript{−} and I\textsuperscript{−} were poor bioluminescence reagents despite their well-documented reactivity with MPO. (Figure 2.5) This suggested that L-012 should not be considered a general reporter of MPO activity and, from this, a bioluminescent assay system for MPO detection may require different optimal conditions from those published for MPO activity measured by other means. This is well-illustrated when considering solution pH as an assay parameter.
Luminol is known to be highly chemiluminescent in the presence of $\text{H}_2\text{O}_2$ in alkaline solutions, but the effects of pH on L-012 sensitivity to $\text{H}_2\text{O}_2$ and other ROS has not been published. Unlike luminol, L-012 will begin to oxidize even absence of $\text{H}_2\text{O}_2$ at pH $> 5$; addition of $\text{H}_2\text{O}_2$ causes further MPO-independent chemiluminescence detrimental to assay specificity. (Figure 2.8C) Although such an adequately acidic environment to prevent background chemiluminescence also favors the MPO halogenation cycle responsible for its bioluminescence, literature suggests that probe oxidation by hypohalous acids is also impeded at low pH.$^{46,50,53}$ Further complicating the issue is decreased solubility of L-012 in acidic buffers; restrictions on probe concentration must also be considered when optimizing an assay parameter as superficially straightforward as pH. (Figure 2.9C) Not only does reporter precipitation add experimental variance to any enzyme activity assay, but L-012 additionally forms a visible film at the air-liquid interface as it crashes out of solution. The local concentration of L-012 is very high in this film, which is also exposed to molecular oxygen from ambient air. Numerous experiments were prematurely terminated when L-012 was used too near to its solubility limit in acidic buffers, causing stochastic “flashing” in random microtiter plate wells which were bright enough to saturate the camera and wash out all adjacent signal.

$\text{H}_2\text{O}_2$ is also more nuanced than substrates in classic enzyme assay systems; not only can $\text{H}_2\text{O}_2$ directly oxidize L-012 and reduce assay specificity, but it can also inhibit the halogenation reaction for which it is a substrate (see Figure 1.3 on page 13). As Figure 2.7B illustrates, MPO-dependent bioluminescence is optimized in a narrow concentration of $\text{H}_2\text{O}_2$, above which the MPO solutions are indistinguishable from their buffer controls. As other reaction parameters are adjusted to maintain halogenation and reduce MPO-independent L-012 oxidation, increasing $\text{H}_2\text{O}_2$ concentrations can be used while maintaining
MPO resolution. (Figure 2.10A and Figure 2.10B) Although these experiments demonstrate that reaction conditions can be manipulated to allow for robust linear MPO bioluminescence with L-012, the discussion to this point has not considered a crucial component of a plasma MPO activity assay.

As Figure 2.10 illustrates, optimization of MPO bioluminescence conditions in buffer is insufficient for a plasma activity assay. Whole plasma is a complex mixture containing many potent inhibitors of MPO activity and consequent bioluminescence. (Figure 2.11A) Although macromolecular inhibitors such as ceruloplasmin have been identified in the literature, most of the quenching activity of plasma is found in the <5 kDa fraction.\textsuperscript{54–58} (Figure 2.11B) This agrees with the potent inhibition demonstrated by ascorbic acid; although it is one of many small-molecule antioxidants in blood plasma, it alone is sufficient at physiologic concentration to inhibit >95% of MPO-dependent bioluminescence in plasma. (Figure 2.12) Published literature suggests that other compounds such as glutathione, cysteine, homocysteine, and methionine are at least as potent in their MPO inhibition.\textsuperscript{59–61} These molecules not only quench nearly all plasma bioluminescence, but represent a matrix of confounding parameters that are highly variable between individuals and across time within individuals. Their effect is further made problematic by the nonlinear relationship between plasma concentration and inhibition; the extent of this nonlinearity is also a function of MPO activity as demonstrated by Figure 2.13C. Dilution of the plasma below the threshold of inhibitory effects was unsuccessful as MPO was also diluted to undetectable concentrations.

ELISAs measuring plasma MPO concentration bypass all of these difficulties through immunospecific MPO isolation. Doing so comes at great cost, both in the monetary cost of commercial ELISA kits and the time required to perform the many steps composing the assays. Although some veterinary assays have been developed to utilize intrinsic MPO activity
after immunologic purification, these approaches still use expensive biologic reagents that limit accessibility to a test this project is intended to apply to CVD, which disproportionately afflicts people with limited access to costly health care.\textsuperscript{62,63}

In conclusion, the work presented in this chapter characterizes many previously unpublished characteristics of the MPO–L-012 bioluminescence system that are important factors in the design and development of a rapid assay for plasma MPO activity. Although complex aspects of the system such as MPO kinetics and substrate/reporter interactions can be exhaustively explored to find optimum conditions for MPO-dependent bioluminescence, the parameter optima are not independent of plasma characteristics that vary widely between samples. The remaining chapters will discuss the inception, optimization, and application of a non-immunologic MPO assay that removes or mitigates confounding plasma components before quantifying MPO activity with L-012 bioluminescence.

\subsection*{2.5 References}


Chapter 3

Myeloperoxidase sequestration on solid substrates

3.1 Introduction

Over the last 20 years, inflammation has become a central component in the understanding of cardiovascular disease (CVD) pathogenesis.\textsuperscript{1–4} As a consequence of this realization, biomarkers of inflammation have joined those indicating congestive heart failure, elevated coagulation cascade activity, and lipid oxidation as risk factors for major adverse cardiovascular events (MACEs) such as myocardial infarction (MI) and stroke.\textsuperscript{5–8} One such indicator of inflammatory leukocyte activation is myeloperoxidase (MPO), an enzyme found primarily in the azurophilic granules of neutrophils. MPO concentration in either plasma or serum has been shown to be a statistically relevant predictor of CVD risk, both for progression to symptomatic disease and sequelae in patients presenting with a MACE.\textsuperscript{9–20} Clinical assays for plasma MPO use immunological methods, primarily the sandwich enzyme-linked immunosorbent assay (ELISA), to isolate MPO from other plasma components that prevent accurate measurement. Most commercial assays rely on colorimetric oxidation of reporter dyes by horseradish peroxidase (HRP) conjugated to secondary or tertiary antibodies instead of utilizing native MPO activity.
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Introduction

MPO serves a physiological role in non-specific host defense by generating reactive oxygen species (ROS), mostly in the form of hypochlorous acid (HOCl), at microbicidal concentrations in phagosomes containing foreign microbes.\textsuperscript{21} MPO activity is non-specific; ROS can damage host-biomolecules in the case of pathological inflammation such as that found in atherosclerosis and other chronic disease processes.\textsuperscript{22} It comes as little surprise that free MPO circulating through the body is inhibited, both by small-molecule antioxidants and specific protein inhibitors such as ceruloplasmin.\textsuperscript{23–27} This was confirmed in the previous chapter as whole plasma proved a potent inhibitor of MPO halogenation activity as read by L-012. A bioluminescence assay thus requires a method to isolate MPO from the inhibitory components in plasma. ELISAs accomplish this with anti-MPO immunoglobulins adsorbed onto microtiter plates. A small body of literature investigates the concept of isolating MPO in same manner as ELISA,\textsuperscript{1} but utilizing the intrinsic peroxidase activity to measure bound protein in lieu of immunoconjugated HRP.\textsuperscript{28,29} The experiments described in this chapter aimed at taking this simplified strategy further by completely eliminating immunoglobulins from the assay. Solution conditions were optimized for selective retention of MPO from whole plasma, disruption of MPO/inhibitor interactions, and L-012 bioluminescence sensitivity and specificity for MPO activity. Additional experiments were conducted to find a suitable bioluminescence standard curve against which human plasma samples with unknown MPO can be calibrated. The resulting assay technique, referred to as the ‘MPO activity on a polymer surface (MAPS) assay,’ is tested against commercially available ELISAs in Chapter 4.

\textsuperscript{1}\textnormal{in order to better diagnose and treat inflammatory diseases in horses}
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3.2.1 Reagents and materials

Lyophilized L-012 was provided by Wako Chemicals USA (Richmond, Virginia; product number 120-04891) was resuspended to 16.1 mM in ultrapure water and stored in 200 µL aliquots at −20 °C. Lyophilized human MPO from EMD Millipore/Calbiochem (Darmstadt, Germany; product number 475911) was resuspended in pH = 6.0 buffer containing 100 mM NaCl and 50 mM acetate, as recommended by the supplier, with final concentration 100 mg/L. Single-use 10 µL aliquots were stored at −20 °C. Lyophilized, citrated bovine plasma from Sigma Aldrich (Saint Louis, Missouri; product number P4639) was resuspended to the original 10 mL with ultrapure water and gently agitated at room temperature until particulates had dispersed. Reconstituted bovine plasma (RBP) was stored at −20 °C. All other reagents were supplied by Sigma Aldrich.

Saturated ammonium sulfate ((NH₄)₂SO₄) was prepared by warming ultrapure water in a 37 °C water bath and incrementally adding solid (NH₄)₂SO₄ until solvation was not complete within 10 min. The solution was then allowed to cool overnight to room temperature, resulting in visible crystals on the floor of the glass bottle. Using a weighted average of published (NH₄)₂SO₄ solubility at various temperatures, the saturated solution was declared as 4.05 M (NH₄)₂SO₄ in all subsequent calculations.³⁰

The Bio-Dot vacuum filtration apparatus (Bio-Rad Laboratories; Hercules, California; product number 170-6545) was graciously provided by Dr. Jason Weber. Nitrocellulose membranes were supplied by GE Osmonics, Inc. (Schenectady, New York; product number EP4HY00010). Polyvinylidene fluoride (PVDF) membranes were provided by EMD Millipore (Billerica, Massachusetts; product number IPVH00010). Nitrocellulose and PVDF filter plates
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were supplied by Pall Corporation (Port Washington, New York; product numbers 5022 and 5026). Polystyrene adsorption experiments were performed using 96-well, black-walled, flat-bottom, tissue culture-treated polystyrene microtiter plates (Corning Inc.; Corning, New York; product number 3603).

3.2.2 Bioluminescence imaging

Bioluminescence images were obtained with the ONYX/M imaging system with a XR/MEGA-10Z camera (Stanford Photonics; Palo Alto, California). Images were obtained with 10 s integrations to prevent camera saturation and combined into 1 min images with the Fiji distribution of ImageJ image analysis software.31 ONYX data were either gathered as raw luminescence or counts depending on hardware capability, as noted in detailed experiment methods.

3.2.3 Data analysis and statistics

Images were quantified with the Fiji distribution of ImageJ image analysis software.31 Data from the ONYX/M were processed to correct for optical vignetting as detailed in the Appendix Section §7.4.2 on page 229. Regions of interest (ROIs) were created in microtiter plate arrays using the Quadrangular ROI array program, the source code of which can be found on page 226. Quantifications were normalized to image ROI pixel area to account for inter-experiment differences in ROI layout. All statistical analyses were performed in IBM SPSS Statistics 21 (Armonk, New York) unless otherwise noted. The novel weighted least squares (WLS) regression method is explained in detail in Appendix Section §7.2 and the code for implementing the method can be found on page 212. Nonlinear regression confidence bands were calculated with the delta method.32
3.2.4 Human plasma samples

Human blood was obtained from the cephalic vein of healthy volunteers into K$_2$EDTA tubes for plasma isolation. Plasma was isolated by two-stage centrifugation at 4 °C: 1,000 × g in the collection tubes for 15 min followed by 14,000 × g in microcentrifuge tubes for 10 min. Plasma was stored at −20 °C and thawed at 4 °C before use. The Institutional Review Board at Washington University in St. Louis approved these study protocols.

3.2.5 Passive membrane binding

Images shown in Figure 3.1A was obtained by serially diluting MPO into pH = 6.0 DPBS with 5 mM acetic acid (AcDPBS). Each dilution was applied in 0.5 µL droplets to a dry nitrocellulose membrane. The membrane was immersed in AcDPBS on an orbital mixer for 13 min before immersion for imaging in a solution containing 500 µM L-012 and 10 mM hydrogen peroxide (H$_2$O$_2$) and integrating raw luminescence for 1 min in the ONYX/M.

Image shown in Figure 3.1C and quantified in Figure 3.1D were obtained by first washing the MPO residue from a consumed stock tube with heat-inactivated fetal bovine serum (∆FBS). A microtiter plate was arrayed in a diagonal pattern of the unknown MPO ∆FBS at 100, 10, or 0% v/v in AcDPBS. A prototype membrane loading cassette was assembled with this plate as shown in Figure 3.1B by layering a nitrocellulose membrane, a sponge pad used in western blot cassettes, and a discarded micropipette tip rack over the wells and securing with elastic straps and aluminum spring clamps. The cassette was inverted, gently tapped on a benchtop, and left standing for 10 min before the membrane was removed and washed in AcDPBS for 10 min. The membrane was immersed in AcDPBS containing 10 mM H$_2$O$_2$ and 1 mM luminol and imaged for raw luminescence in the ONYX/M.
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Data from PVDF filter plates shown in Figure 3.2 were obtained by diluting MPO into AcDPBS. A PVDF plate was pre-treated with methanol and rinsed three times with AcDPBS before loading wells with 50 µL of the MPO dilutions. After a 5 min (luminol plates) or 30 min (L-012 plates) incubation, MPO was aspirated from the wells and three 250 µL AcDPBS washes removed unbound MPO. The plate was then imaged in the ONYX/M with 80 µL AcDPBS-based imaging solution containing 10 mM H₂O₂ and either 1 mM luminol or 500 µM L-012.

Nitrocellulose plates were similarly imaged in the presence or absence of human plasma as shown in Figure 3.3. MPO was diluted to 2.5 µg/L into Dulbecco’s phosphate buffered saline (DPBS) or 50 µg/L into fresh citrated human plasma collected from a healthy volunteer, which was then diluted to 5% v/v into DPBS. Nitrocellulose wells received 100 µL DPBS ± plasma and ±MPO for 30 min while shaking at 850 rpm. Each well was washed three times with 300 µL DPBS and imaged with either 500 mM NaCl DPBS with 200 µM H₂O₂ and 100 µM L-012 (Figure 3.3A) or DPBS adjusted to pH = 6.0 with 200 µM H₂O₂ and 100 µM L-012 (Figure 3.3B). Data shown are raw luminescence from the ONYX/M quantified at the 2 min timepoint of each imaging sequence.

3.2.6 Vacuum cassette MPO assay

Vacuum cassette data shown in Figure 3.4A and Figure 3.4B were obtained by serially diluting MPO into DPBS at the concentrations shown. A nitrocellulose membrane in the Bio-Dot apparatus was prepared by filtering 100 µL DPBS through each of the wells before similarly filtering 100 µL of each MPO solution. Each well was washed with 400 µL DPBS and vacuum pressure was maintained until each well was dry. The cassette was imaged with the membrane in place by adding 200 µL DPBS with 10 µM L-012 and 100 µM H₂O₂. Raw luminescence
for the time sequence and the 2 min timepoint are shown. Data from the 2 min timepoint could not be fit to equation (3.1) due to high variance.

A similar protocol was used to image MPO diluted into filtered ΔFBS before loading onto nitrocellulose as shown in Figure 3.4C and Figure 3.4D. Endogenous MPO remaining in the ΔFBS after heating was removed via 50 kDa cutoff ultrafiltration (Corning, Inc; product number 431485) at 4 °C, 10,000 rpm for 80 min. Serial MPO dilutions made into whole, filtered ΔFBS were diluted to 1% v/v in DPBS; 500 µL of each dilution was added to wells over a nitrocellulose membrane in the vacuum cassette. After the ΔFBS solutions were filtered through the membrane, each well was washed with 300 µL DPBS and vacuum pressure was maintained until each well was dry. The cassette was imaged with the membrane in place by adding 200 µL DPBS with 10 µM L-012 and 100 µM H₂O₂. Raw luminescence for the time sequence and the 2 min timepoint are shown. Data from the 2 min timepoint were fit to the equation

\[ B = \frac{B_{\text{max}}}{1 + e^{H([\text{MPO}] - D_{50})}} \]  

where MPO is quantified by the pre-dilution concentration in whole, filtered ΔFBS, \( H \) is the Hill coefficient, \( D_{50} \) is the half-saturation concentration, and \( B_{\text{max}} \) is the asymptotic maximum bioluminescence signal.

### 3.2.7 MPO adsorption onto tissue culture-treated polystyrene

Adsorption of MPO from buffer and human plasma shown in Figure 3.5 was investigated by diluting fresh human plasma collected in K₂EDTA vacuum phlebotomy tubes to 0.5% v/v in calculated pH (\( \text{pH} = 6.5 \) buffer containing 6.28 mM Na₂HPO₄, 18.7 mM NaH₂PO₄, and 130 mM NaCl. Serial dilutions of MPO accounting for the 0.5% v/v dilution were made into this buffer ± diluted human plasma. Tissue culture treated microtiter plates were arrayed
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with 50 µL of each MPO dilution in quadruplicate then incubated for 30 min on a 1,000 rpm orbital shaker. Each well was aspirated dry and washed three times with 150 µL dilution buffer and a final time with 300 µL buffer before receiving 150 µL C\(\text{pH} = 6.0\) imaging solution containing 200 µM \(\text{H}_2\text{O}_2\), 50 µM L-012, 2.28 mM \(\text{Na}_2\text{HPO}_4\), 22.7 mM \(\text{NaH}_2\text{PO}_4\), 500 mM \(\text{NaCl}\), and 20 mM \(\text{NaBr}\). Plates were shaken for 1 min at 1,000 rpm before imaging photon counts in the ONYX/M. Calibration curves from the 5 min timepoint were used for WLS regression. MPO is annotated with pre-dilution concentrations in whole plasma; the same adjustment was made in annotating MPO in pure buffer.

MPO adsorption kinetics onto polystyrene as shown in Figure 3.6 were measured by serially diluting MPO into \(\Delta\text{FBS}\) and further diluting this to 0.5% \(v/v\) in a \(C\text{pH} = 6.5\) buffer containing 130 mM \(\text{NaCl}\), 6.30 mM \(\text{Na}_2\text{HPO}_4\), and 18.7 mM \(\text{NaH}_2\text{PO}_4\). Each MPO concentration was added in triplicate to a tissue culture-treated plate at 0, 35, 65, 90, 110, 125, 135, and 140 min timepoints of 1,000 rpm orbital shaking duration. Immediately after the final addition, wells were aspirated dry and washed four times with 150 µL dilution buffer. Each well then received 150 µL \(C\text{pH} = 6.0\) imaging solution containing 200 µM \(\text{H}_2\text{O}_2\), 50 µM L-012, 500 mM \(\text{NaCl}\), 20 mM \(\text{NaBr}\), 2.28 mM \(\text{Na}_2\text{HPO}_4\), and 22.7 mM \(\text{NaH}_2\text{PO}_4\). The plates were shaken for 2 min at 1,000 rpm and imaged for photon count quantification in the ONYX/M. Counts from the 5 min timepoint were used in WLS regression with pre-dilution MPO concentrations.

The effects of anticoagulation were investigated as shown in Figure 3.7 by obtaining fresh plasma from the same healthy volunteer using \(\text{K}_2\text{EDTA}\) and lithium heparin phlebotomy tubes. MPO was serially diluted into 1% \(v/v\) plasma with each anticoagulant, accounting for dilution into DPBS, and 50 µL of each MPO concentration was loaded in triplicate into wells of a tissue culture-treated microtiter plate. After a 30 min incubation with 1,000 rpm
orbital shaking, wells were aspirated dry and washed four times with 300 µL DPBS. Wells were then treated with 150 µL C\text{pH} = 6.0 imaging solution containing 200 µM H\textsubscript{2}O\textsubscript{2}, 50 µM L-012, 20 mM NaBr, 2.28 mM Na\textsubscript{2}HPO\textsubscript{4}, and 22.7 mM NaH\textsubscript{2}PO\textsubscript{4}, shaken briefly up to 1,000 rpm, and imaged with photon count quantification in the ONYX/M. Data from the 5 min timepoint were analyzed with WLS regression using pre-dilution MPO concentrations.

ΔFBS and human plasma were directly compared as MPO adsorption carriers by serially diluting MPO into whole ΔFBS or human plasma from a healthy volunteer, then diluting 2 µL each of the sixteen solutions into 500 µL C\text{pH} = 6.5 buffer containing 130 mM NaCl, 6.29 mM Na\textsubscript{2}HPO\textsubscript{4}, and 18.7 mM NaH\textsubscript{2}PO\textsubscript{4} in 900 µL polypropylene cluster tubes. After mixing vigorously on a vortex shaker, 100 µL of each MPO concentration in carrier plasma was arrayed onto a tissue culture-treated microtiter plate, which was then shaken at 1,000 rpm for 30 min. Wells were then aspirated dry and washed twice with 150 µL dilution buffer and once more with 300 µL before receiving 150 µL C\text{pH} = 6.0 imaging solution containing 100 µM H\textsubscript{2}O\textsubscript{2}, 50 µM L-012, 200 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 20 mM NaBr, 3.09 mM citric acid, and 21.9 mM trisodium citrate. The plate was shaken briefly up to 1,000 rpm on an orbital shaker and imaged in the ONYX/M to quantify raw luminescence as shown in Figure 3.8. The 5 min timepoint of each carrier series was analyzed with WLS regression separately; the human plasma series was also calibrated against the ΔFBS standards by WLS inverse regression. Human plasma was compared to RBP as shown in Figure 3.9 with the same protocol with the addition of 50 ppm \textit{v/v} polysorbate 20 (Tween20) in the plasma dilution buffer and 100 ppm \textit{v/v} Tween20 in the imaging solution.

Microfiltration was used to fractionate RBP based on filter cutoffs to determine if adsorption depended on micro- or macro-molecular interactions, demonstrated in Figure 3.10. Centrifugal ultrafiltration concentrators (Corning Life Sciences Spin-X tubes with 5 kDa,
30 kDa, and 100 kDa filtration membranes, product numbers 431482, 431484, and 431486) were prepared by filtering 6 mL ultrapure water for 20 min at 4 °C, 4,000 × g to remove contaminants from the manufacturing process. RBP (30 µL, equal to the retention volume of the feeder reservoirs) was diluted to 5% v/v into pH = 6.5 buffer containing 130 mM NaCl, 6.28 mM Na_{2}HPO_{4}, and 18.7 mM NaH_{2}PO_{4}. Filter tubes were spun for 75 min at 4 °C, 4,000 × g. The retained 30 µL of feeder solution was re-diluted with 6 mL of the dilution buffer and mixed thoroughly to make the matching >cutoff solutions. From these, 0–300 µg/L MPO solutions were made accounting for the 0.5% v/v RBP dilutions. Each sample was loaded as quadruplicate 100 µL volumes into tissue culture-treated microtiter plate wells, which were shaken at 1,000 rpm for a 30 min incubation. Wells were then aspirated dry and washed twice with 150 µL dilution buffer and once with 300 µL before receiving 150 µL pH = 6.0 imaging solution containing 100 µM H_{2}O_{2}, 50 µM L-012, 200 mM (NH_{4})_{2}SO_{4}, 20 mM NaBr, 3.09 mM citric acid, and 21.9 mM trisodium citrate. The 5 min timepoint of each filtrate×MPO treatment was analyzed with WLS regression. The same incubation protocol, pH = 6.5 dilution buffer, and pH = 6.0 imaging solutions were used to test bovine serum albumin (BSA) and human fibronectin as adsorption carriers shown in Figure 3.11. An MPO dilution factor of 0.2% v/v was used when measuring BSA co-adsorption and 0.4% v/v was used with fibronectin.

3.2.8 Screening MPO adsorption and imaging conditions

A small screen of adsorption conditions shown in Figure 3.12 was performed with numbered solutions listed in Table 3.1. Two microcentrifuge tubes received 90 µL of each solution 1–16, into which 10 µL EDTA-anticoagulated human plasma ±100 µg/L MPO was added. Tubes containing solutions intended to precipitate plasma components (5–15) were centrifuged.
at 4 °C, 14,000 × g for 30 min; the remaining tubes were refrigerated at 4 °C for 30 min. From these 10% v/v dilutions or supernatants, 5 µL was added to 495 µL of the matching solution number (parenthetical solutions in Table 3.1 in the case of supernatants) resulting in a final dilution factor of 0.1% v/v. Each of the 32 treatment × MPO conditions was loaded in triplicate 50 µL volumes onto a tissue culture-treated microtiter plate, which was orbitally shaken for 20 min at 1,000 rpm. Each well was aspirated dry and washed four times with 150 µL of the same buffer used for the final dilution stage. After a final dry aspiration, each well received C\(\text{pH} = 6.0\) imaging solution containing 100 µM H\(_2\)O\(_2\), 50 µM L-012, 200 mM (NH\(_4\))\(_2\)SO\(_4\), 20 mM NaBr, 2.28 mM Na\(_2\)HPO\(_4\), and 22.7 mM NaH\(_2\)PO\(_4\). The imaging solution was shaken at 1,000 rpm on the plate for 1 min before imaging in the ONYX/M. Signal ratios ±95% confidence intervals were calculated using log-linked gamma regression performed on photon counts from the 5 min timepoint.

A pH survey for optimal adsorption was performed to investigate the findings from the loading solution screen, as shown in Figure 3.13. Eight solutions with 130 mM NaCl and total phosphate concentrations of 25 mM spanning C\(\text{pHs} = 6–8\) were used to dilute RBP supplemented with 0, 100, or 300 µg/L human MPO to 0.4% v/v. Each of these solutions was arrayed as 75 µL volumes in triplicate on a tissue culture-treated microtiter plate and incubated for 30 min with 1,000 rpm orbital shaking. Each well was aspirated dry and washed with the corresponding C\(\text{pH}\) dilution buffer: twice with 150 µL and a third time with 300 µL. After a final dry aspiration, each well received 150 µL of an imaging solution, C\(\text{pH} = 6.0\), containing 100 µM H\(_2\)O\(_2\), 50 µM L-012, 200 mM (NH\(_4\))\(_2\)SO\(_4\), 20 mM NaBr, 3.09 mM citric acid, and 21.9 mM trisodium citrate. The plate was shaken briefly up to 1,000 rpm on an

\(^{2}\)As some of these solutions contained (NH\(_4\))\(_2\)SO\(_4\), aspiration was not done into the conventional, bleached vacuum flask. (A lesson learned the hard way\(^{33}\) A disposable, vacuum-safe biohazard tube was constructed from conical tubes and paraffin film to be autoclaved at the conclusion of this screen.
orbital shaker and imaged in the ONYX/M to quantify raw luminescence. Data from the 5 min timepoint were used for WLS regression with pre-dilution MPO concentrations.

A similar screen of imaging conditions shown in Figure 3.14 was performed with solutions numbered in Table 3.2. Human plasma ±100 µg/L pre-dilution MPO was diluted to 1% v/v in C_pH = 6.5 buffer containing 130 mM NaCl, 6.29 mM Na_2HPO_4, and 18.7 mM NaH_2PO_4. Each plasma series was arrayed in 50 µL volumes into 48 of 96 wells on a tissue culture-treated microtiter plate which was shaken for 15 min at 1,000 rpm. Each well was aspirated dry and washed four times with 150 µL of the dilution buffer. After a final dry aspiration, triplicate wells of each plasma series received 150 µL of one of 16 imaging solutions. The screened solutions were shaken at 1,000 rpm on the plate for 1 min before imaging in the ONYX/M. Signal ratios ±95% confidence intervals were calculated using log-linked gamma regression performed on photon counts from the 5 min timepoint.

### 3.2.9 Enhancing adsorbed MPO bioluminescence

The effect of (NH_4)_2SO_4 observed in the imaging solution screen was further investigated in experiments shown in Figure 3.15. A titration of (NH_4)_2SO_4 on adsorbed MPO from human plasma was performed by preparing 0, 100, and 300 µg/L MPO in C_pH = 6.5 buffer containing 200 ppm v/v Tween20, 130 mM NaCl, 6.28 mM Na_2HPO_4, and 18.7 mM NaH_2PO_4. This MPO along with human plasma were diluted 0.4% v/v into a C_pH = 6.5 plate loading buffer containing 50 ppm v/v Tween20, 130 mM NaCl, 6.28 mM Na_2HPO_4, and 18.7 mM NaH_2PO_4. Each of the three plasma ± MPO solutions was arrayed as 75 µL volumes evenly into a microtiter plate, which was shaken at 1,000 rpm for 30 min. Each well was aspirated dry and washed thrice with 150 µL of the plate loading buffer. After a final dry aspiration, each well received one of eight imaging solutions with varying (NH_4)_2SO_4 along with 3.05 mM citric
acid and 22.0 mM trisodium citrate (CpH = 6.03) with 100 µM H₂O₂, 50 µM L-012, 20 mM NaBr, and 100 ppm v/v Tween20. The plates were shaken briefly up to 1,000 rpm on an orbital shaker and imaged in the ONYX/M to quantify raw luminescence. Data from the 5 min timepoint were used for regression with the equation

\[ B = B_{\text{min}} + \frac{(B_{\text{max}} - B_{\text{min}}) \cdot [(\text{NH}_4)_2\text{SO}_4]^H}{[(\text{NH}_4)_2\text{SO}_4]^H + EC_{50}^H} \] (3.2)

where \( EC_{50} \) is the concentration of half-maximal enhancement effect, \( H \) is the Hill coefficient responsible for the sigmoid shape of the titration curve, and \( B_{\text{max}} \) and \( B_{\text{min}} \) are the asymptotic maximum and minimum bioluminescence for each MPO concentration.

The enhancer effect was then quantified for (NH₄)₂SO₄ applied to MPO in solution with whole plasma as shown in Figure 3.15B. Healthy-donor plasma supplemented with 0, 100, or 300 µg/L MPO was diluted to a final reaction concentration of 400 ppm v/v in CpH = 6.0 buffer containing 3.05 mM citric acid, 22.0 mM trisodium citrate, and 20 mM NaBr. Serial dilutions of (NH₄)₂SO₄ were made with constant, final concentrations of 50 µM L-012 and 100 µM H₂O₂. A microtiter plate was evenly arrayed with 10 µL of each diluted plasma. The reaction was initiated by adding 100 µL of each (NH₄)₂SO₄ imaging solution and shaking briefly up to 1,000 rpm on an orbital shaker before imaging in the ONYX/M. Raw luminescence data from the 5 min timepoint were fit to the equation

\[ B = B_{\text{min}} + \frac{(B_{\text{max}} - B_{\text{min}}) \cdot [(\text{NH}_4)_2\text{SO}_4] \cdot [(\text{NH}_4)_2\text{SO}_4]}{[(\text{NH}_4)_2\text{SO}_4] + EC_{50}^H} \] (3.3)

lacking a Hill coefficient \( H \) as there was no statistically significant sigmoidal nature to the titration. This protocol was modified with Na₂SO₄ used in place of (NH₄)₂SO₄ as a negative control for NH₄⁺ as shown in Figure 3.15D, the data from which failed to fit the signal enhancement model equations.

\(^3\)calculation does not consider effects of acidic (NH₄)₂SO₄
The effect of (NH₄)₂SO₄ on pure MPO was also investigated as shown in Figure 3.15C. Serial dilutions of (NH₄)₂SO₄ were prepared in a \( \text{CPH} = 6.0 \) buffer containing 10 mM NaBr, 3.05 mM citric acid, and 22.0 mM trisodium citrate. Each of these solutions was arrayed as 75 µL volumes along with 50 µL of the buffer ±500 ng/L final concentration MPO. The reaction was initiated by the final addition of 150 µL buffer containing final concentrations 100 µM H₂O₂ and 30 µM L-012 before immediate imaging in the ONYX/M. As previously, data from the 5 min timepoint were quantified.

The detergent Tween20 was tested for its effects on various portions of the adsorbed MPO bioluminescence assay as shown in Figures 3.17-3.19. To test the effect of Tween20 in the imaging solution, RBP with varying MPO was diluted to 0.4% \( v/v \) in \( \text{CPH} = 6.5 \) buffer containing 130 mM NaCl, 6.28 mM Na₂HPO₄, and 18.7 mM NaH₂PO₄. Each of the four MPO solutions was arrayed as 75 µL volumes evenly into a microtiter plate, which was shaken at 1,000 rpm for 30 min. Each well was aspirated dry and washed twice initially with 150 µL of the plate loading buffer and finally with 300 µL. After a final dry aspiration, each well received one of six \( \text{CPH} = 6.0 \) imaging solutions with varying Tween20, 3.05 mM citric acid, 22.0 mM trisodium citrate, 100 µM H₂O₂, 50 µM L-012, and 20 mM NaBr. The plate was shaken briefly up to 1,000 rpm on an orbital shaker before imaging in the ONYX/M. The 5 min timepoint of each Tween20 treatment was analyzed with WLS regression using pre-dilution MPO concentrations.

This protocol was modified to test the effect of Tween20 in the incubation buffer. Tween20 was serially diluted into the \( \text{CPH} = 6.5 \) buffer containing 130 mM NaCl, 6.28 mM Na₂HPO₄, and 18.7 mM NaH₂PO₄. 500 µL of each Tween20 buffer was added to three cluster tubes, which then received 2 µL undiluted RBP supplemented with 0, 100, or 300 µg/L human MPO. After vigorous vortex mixing, each of the 24 MPO×Tween20 solutions was arrayed
as quadruplicate 75 µL volumes into a tissue culture-treated plate, which was incubated for 30 min with 1,000 rpm orbital shaking. Each well was aspirated dry and washed twice initially with 150 µL of the phosphate buffer (without Tween20) and finally with 300 µL. After a final dry aspiration, each well received C_pH = 6.0 imaging solution with 100 ppm v/v Tween20, 3.05 mM citric acid, 22.0 mM trisodium citrate, 100 µM H₂O₂, 50 µM L-012, and 20 mM NaBr. The plate was shaken briefly up to 1,000 rpm on an orbital shaker before imaging in the ONYX/M. The 5 min timepoint of each Tween20 treatment was analyzed with WLS regression using pre-dilution MPO concentrations.

The third test of Tween20 effects involved the MPO pre-dilution buffer. First, Tween20 was serially diluted into the C_pH = 6.5 buffer containing 130 mM NaCl, 6.28 mM Na₂HPO₄, and 18.7 mM NaH₂PO₄. With each from this series of eight Tween20 buffers, three MPO serial dilutions were made. RBP was diluted to 0.4% v/v in a 50 ppm v/v Tween20 variant of the phosphate buffer; 500 µL of this solution was added to each of 24 cluster tubes along with 2 µL of a single Tween20×MPO solution. After vigorous vortex mixing, each of the 24 cluster tubes was used to array a microtiter plate with triplicate 75 µL volumes. Each well was aspirated dry and washed twice initially with 150 µL of the phosphate buffer (with 50 ppm v/v Tween20) and finally with 300 µL. After a final dry aspiration, each well received C_pH = 6.0 imaging solution with 100 ppm v/v Tween20, 3.05 mM citric acid, 22.0 mM trisodium citrate, 100 µM H₂O₂, 50 µM L-012, and 20 mM NaBr. The plate was shaken briefly up to 1,000 rpm on an orbital shaker before imaging in the ONYX/M. The 5 min timepoint of each Tween20 treatment was analyzed with WLS regression using pre-dilution MPO concentrations.
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3.2.10 Bioluminescence robustness and specificity for MPO

RBP was supplemented with varying concentrations of ascorbic acid to confirm that the previously observed inhibition had been mitigated by the adsorption procedure. First, MPO was serially diluted to 0, 100, and 300 µg/L in a pH = 6.5 buffer containing 200 ppm v/v Tween20, 130 mM NaCl, 6.28 mM Na₂HPO₄, and 18.7 mM NaH₂PO₄. Serial dilutions of ascorbic acid and a 0.4% v/v RBP dilution were made in a 50 ppm v/v Tween20 variant of the same buffer. Cluster tubes were used to combine 300 µL diluted RBP, 10 µL MPO solutions, and 100 µL ascorbic acid titration solutions, all of which corresponded to plasma diluted to 0.4% v/v. Each of the 24 MPO×ascorbic acid solutions was arrayed as quadruplicate 75 µL volumes and incubated with 1,000 rpm orbital shaking for 30 min. The plate was then aspirated dry and washed twice with 150 µL and once with 300 µL of the 50 ppm v/v Tween20 buffer variant. After a final dry aspiration, each well received 75 µL of an imaging solution, pH = 6.0, containing 100 ppm v/v Tween20, 100 µM H₂O₂, 50 µM L-012, 200 mM (NH₄)₂SO₄, 20 mM NaBr, 3.09 mM citric acid, and 21.9 mM trisodium citrate and was shaken briefly up to 1,000 rpm before imaging in the ONYX/M. Data from the 5 min timepoint are shown in Figure 3.16 with all concentrations adjusted to their pre-dilution levels.

Bioluminescence specificity for MPO activity was investigated with the specific inhibitor 4-aminobenzoic hydrazide (4-ABH). Serial dilutions of MPO were prepared in the same buffer used in the ascorbic acid experiments described in the previous method description. Similarly, the MPO solutions along with RBP were diluted to 0.4% v/v into a 50 ppm v/v Tween20 variant of the MPO dilution buffer. Each of the three MPO/RBP solutions was arrayed evenly as 75 µL volumes and incubated with 1,000 rpm orbital shaking for 30 min. The plate was then aspirated dry and washed as described above. Imaging solutions were made with varying 4-ABH concentrations in pH = 6.0 buffer containing 100 ppm v/v Tween20, 100 µM


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H₂O₂, 50 µM L-012, 200 mM (NH₄)₂SO₄, 20 mM NaBr, 3.09 mM citric acid, and 21.9 mM trisodium citrate. After a final aspiration, 75 µL of the imaging solutions were arrayed evenly among the different adsorbed MPO concentrations and imaged as described above. Data from the 5 min timepoint were regressed with the model

\[ B = \frac{B_{\text{max}}IC_{50}^H}{[4-\text{ABH}]^H + IC_{50}^H} \]  \hspace{1cm} (3.4)

where \( H \) is the Hill coefficient, \( IC_{50} \) is the 4-ABH concentration of half-inhibition, and \( B_{\text{max}} \) is the asymptotic maximum bioluminescence. 95% confidence bands were computed with the delta method.

A similar procedure was used to block MPO with another cationic protein, protamine sulfate. As previously, RBP and MPO were diluted into 50 and 200 ppm \( v/v \) Tween20 buffers described above, respectively. Additionally, protamine sulfate was serially diluted into the 200 ppm \( v/v \) Tween20 variant. Each of 24 cluster tubes then received 490 µL RBP (final dilution 0.4% \( v/v \)), 5 µL of one of the three MPO concentrations, and 5 µL of one of the eight protamine solutions. Each of the 24 solutions was arrayed evenly as 75 µL volumes and incubated with 1,000 rpm orbital shaking for 30 min. Imaging solutions were made with \( CPH = 6.0 \) buffer containing 100 ppm \( v/v \) Tween20, 100 µM H₂O₂, 50 µM L-012, 200 mM (NH₄)₂SO₄, 20 mM NaBr, 3.09 mM citric acid, and 21.9 mM trisodium citrate. The plate was washed and imaged as described above. Data from the 5 min timepoint were regressed with the model

\[ B = \frac{B_{\text{max}}IC_{50}^H}{[\text{protamine sulfate}]^H + IC_{50}^H} \]  \hspace{1cm} (3.5)

similar to the 4-ABH analysis using final, in-well protamine concentrations from the incubation step. 95% confidence bands were computed with the delta method.
3.3 Results

3.3.1 Passive MPO immobilization on polymer membranes

Protein isolation and analysis on membranes is a staple technique in molecular biology. A pilot dot-blot experiment demonstrated the feasibility of isolating MPO on nitrocellulose and imaging its intact activity via L-012 bioluminescence. A range of MPO concentrations in buffer spotted onto a nitrocellulose membrane remained visible when immersed in a solution of L-012 and H₂O₂ after first incubating in clean buffer, demonstrating that MPO is bound by nitrocellulose with retained activity. The signal distribution was uneven at each spot, making precise quantification difficult. (Figure 3.1A) A simple, disposable cassette was designed to solve this problem using common labware. Holes in plastic tip racks, which are normally lab waste, are spaced evenly with 96-well microtiter plates, providing pressure around the rims of plate wells. (Figure 3.1B) ΔFBS used to resuspend MPO residue from a stock tube was diluted into AcDPBS and distributed into a microtiter plate to test the apparatus for cross-contamination and MPO binding consistency. After inverting the loaded cassette 10 min and washing the membrane for an additional 10 min, imaging with 1 mM luminol and 10 mM H₂O₂ in AcDPBS revealed even signal distribution with minimal leaking out of each well. (Figure 3.1C) Quantification of the membrane at each well location shows ready discrimination of the two ΔFBS dilutions and the buffer control. (Figure 3.1D) From this proof of concept, commercial products for isolating MPO on solid substrates were considered.

PVDF was considered for its low chemical reactivity, membrane durability, and ready availability as a common protein-binding substrate. Pure human MPO in AcDPBS was loaded into wells of commercial filter plates with PVDF flow-through floors. After incubating for 5 min and washing unbound enzyme, 1 mM luminol and 10 mM H₂O₂ were unable to resolve
Figure 3.1: Passively loading MPO onto nitrocellulose. *(A)* Preliminary demonstration of MPO sequestration and bioluminescence on a nitrocellulose solid substrate. Each spot on the nitrocellulose membrane contains 0.5 µL MPO diluted into AcDPBS. Image shows 1 min integration with 500 µM L-012 and 10 mM H$_2$O$_2$ in the same buffer. *(B)* Schematic for an early prototype membrane loader designed to bind proteins from plasma and allow removal of small-molecule antioxidants. Samples were loaded into a microtiter plate and covered with a nitrocellulose membrane secured by a padded, 96-well tip rack. Metal clamps (not shown) locked the cassette together before inversion to expose the membrane with the contents of the microtiter plate. *(C)* Image of a nitrocellulose membrane loaded with the prototype cassette. Whole ΔFBS, 10% v/v ΔFBS in AcDPBS, or AcDPBS alone were exposed to the membrane for 10 min followed by a 10 min wash incubation in the same buffer. MPO concentration in ΔFBS was not determined in this feasibility experiment. The membrane was immersed in the buffer containing 1 mM luminol and 10 mM H$_2$O$_2$ prior to imaging. *(D)* Quantification of membrane bioluminescence shown in (C). Each line represents individual ROIs.
MPO in the physiological range reported in the literature.\textsuperscript{12} MPO loaded at the highest concentration of 300 µg/L was resolvable, albeit poorly. (Figure 3.2A) A longer incubation of 30 min as well as imaging with L-012 in the place of luminol improved resolution of 100 µg/L MPO, but the lower concentrations were only marginally differentiable from the buffer control. (Figure 3.2B)

MPO is heavily modified with hydrophilic residues and is itself a cationic protein at acidic and neutral pH (see page 10), so nitrocellulose filter plates would hypothetically bind MPO more tightly than would PVDF plates. In general, background luminescence from nitrocellulose filter plates was much higher than that seen with PVDF, even when imaging with 80% lower L-012 and 98% lower H$_2$O$_2$ concentrations. Serial dilutions of plasma supplemented with 50 µg/L MPO into DPBS were poorly resolvable from native
plasma. Serially diluting pure MPO into DPBS free from plasma did not improve resolution. (Figure 3.3A) Although background luminescence from the nitrocellulose plates was reduced by imaging in AcDPBS, neither resolution of pure MPO nor of plasma supplemented MPO was improved over what was seen with neutral DPBS. (Figure 3.3B)

Additional nitrocellulose formats were also tested as MPO assay platforms. These included: nitrocellulose films deposited onto microscopy slides, membrane dipsticks modeled after modern blood glucose tests, nitrocellulose disks incubated in plasma and imaged in microtiter plate wells, and powdered nitrocellulose slurries centrifuged out of plasma suspensions for imaging. Unfortunately, each method was wildly inconsistent and failed to differentiate MPO-spiked plasmas from native controls. (Data not shown)

### 3.3.2 Vacuum-driven MPO immobilization on nitrocellulose

Protein blotting membranes are usually loaded using electromagnetic fields to drive proteins to the membrane surface. This is performed by first denaturing the proteins before they are coated with an ionic detergent such as sodium dodecyl sulfate. In order to maintain native protein function, vacuum pressure was instead used to draw samples through a nitrocellulose membrane loaded into a Bio-Dot apparatus. Pure MPO adhered to the membrane and resulted in better resolution when imaged directly in the Bio-Dot apparatus with 10 µM L-012 and 100 µM H$_2$O$_2$ in DPBS. Unfortunately, the kinetic variability between replicates was consistently high. (Figure 3.4A) Additionally, standard curves constructed with this method were not linear over a wide MPO concentration range. (Figure 3.4B)

To determine if MPO bioluminescence was linear with concentration in plasma, ΔFBS supplemented with pure human MPO was diluted to 1% $v/v$ in DPBS and similarly drawn through a nitrocellulose membrane by a vacuum. Although the final MPO concentrations were
Figure 3.3: MPO bioluminescence in nitrocellulose filter plates. (A) Plasma ± 50 µg/L MPO and serially diluted into DPBS and incubated in a nitrocellulose-bottom microtiter plate. Wells were washed with DPBS and imaged with 200 µM H₂O₂ and 100 µM L-012 in DPBS supplemented with NaCl to a final concentration of 500 mM. Individual replicates (n = 3) are shown from the 2 min timepoint after the imaging solution was added to the plate. (B) Data from a variation of the experiment shown in (A) using DPBS without additional NaCl and adjusted with HCl to pH = 6.0 for the imaging solution. The acidic imaging buffer reduced the high background luminescence from the nitrocellulose plate but also reduced MPO resolution.
matched to those used in pure-buffer membrane loading, ΔFBS-diluted bioluminescence was more consistent. (Figure 3.4C) Although standard curves were reproducible, the non-linear relationship between loaded MPO and bioluminescence remained. (Figure 3.4D)

3.3.3 Co-adsorption of MPO and carrier proteins onto polystyrene

Although used to prepare ELISA plates by tightly adsorbing immunoglobulins with affinity for the desired analyte, imaging high-affinity protein binding plates did not show any MPO-dependent bioluminescence after a wide variety of adsorption incubation conditions. (Data not shown) Although not intended for this purpose, tissue culture-treated polystyrene on a 96-well microtiter plate retained MPO diluted into human plasma. Buffer washes prior to imaging with 100 µM H₂O₂ and 50 µM L-012 in a mildly acidic phosphate buffer mitigated the effects of plasma inhibitors (see page 60). This adsorption required the presence of plasma; pure MPO similarly diluted and incubated in the same buffer did not exhibit the MPO dose-response with L-012 bioluminescence (Figure 3.5A) Although pure MPO adhered to polystyrene was detectable, calibration curves prepared from it were very shallow (5 min limit of detection = 97 µg/L MPO, \( R^2 = 0.847 \)) compared to those from MPO incubated with human plasma (5 min limit of detection = 3.7 µg/L MPO, \( R^2 = 0.995 \)). (Figure 3.5B)

Published ELISA plate adsorption protocols generally call for incubations spanning many hours to reliably retain immunoglobulins; MPO adsorption from ΔFBS was more rapid. Applying ΔFBS supplemented with human MPO and immediately aspirating and washing was adequate to coarsely resolve MPO concentration upon imaging with 50 µM L-012 and 100 µM H₂O₂. (Figure 3.6A) Quantifying the dose response with WLS regression showed that peak MPO resolution is achieved by a 15 min incubation and longer durations did not
Figure 3.4: Vacuum cassette filtration of MPO through nitrocellulose. (A) Bioluminescence time series and (B) 2 min timepoint of purified MPO serially diluted into DPBS and drawn through a nitrocellulose membrane in a Bio-Dot cassette. Cassette wells were washed by drawing DPBS through after the MPO solutions. The membrane was then imaged in the cassette by addition of 10 µM L-012 and 100 µM H₂O₂ in DPBS. MPO is annotated with final concentrations drawn through the membrane. (C) Bioluminescence time series and (D) 2 min timepoint of MPO serially diluted into ΔFBS before further 1:100 dilution into DPBS and similarly loaded onto nitrocellulose. Each well was imaged with 10 µM L-012 and 100 µM H₂O₂. MPO is annotated with pre-dilution concentrations in whole ΔFBS. Regression with equation (3.1), shown as a dashed line ± 95% confidence bands in gray, suggests that this method is only linear between 40–80 ng/L MPO. Raw data (n = 4) are shown in (A)–(C), mean ± standard deviation in (D).
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Results

Figure 3.5: Adsorption of MPO from human plasma onto tissue culture-treated polystyrene.

(A) Bioluminescence time series of MPO ± human plasma incubated on tissue culture-treated polystyrene plates prior to imaging with 50 µM L-012 and 200 µM H₂O₂. Both solution series were diluted to 0.5% v/v in a pH = 6.5 phosphate buffer with 130 mM NaCl. Data are shown as individual replicates (n=4). (B) WLS regression on data from the 5 min timepoint of the time series shown in (A). The limit of detection when plasma was added as an adsorption carrier was 3.7 µg/L MPO with $R^2 = 0.995$, while regression with MPO alone resulted in a limit of 97 µg/L MPO with $R^2 = 0.847$. Data are plotted as mean ± standard deviation. WLS results are shown as dashed lines ± gray 95% confidence bands. Quadruplicate 95% prediction bands are shown as long-dashed lines.

substantially reduce the quality of the calibration curves, which consistently achieved values of $R^2 > 0.995$. (Figure 3.6B)

The anticoagulant used at plasma collection had a dramatic effect on MPO adsorption onto polystyrene. Plasma collected in vacuum phlebotomy tubes coated in lithium heparin reduced adsorbed MPO bioluminescence to a constant baseline level, slightly below that of healthy donor plasma collected in K₂EDTA vacuum tubes. Supplementing this heparinized plasma with exogenous MPO had no effect on resulting bioluminescence. (Figure 3.7A) Unlike pure MPO adsorbed onto the microtiter plates from buffer, which resulted in a poor but quantifiable bioluminescence response (see Figure 3.5B), MPO adsorption from heparinized plasma had no quantifiable correlation with loaded MPO concentration as determined by
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Results

Figure 3.6: Rapid kinetics of MPO adsorption onto polystyrene. (A) MPO diluted into ΔFBS and incubated on tissue culture-treated polystyrene microtiter plates for different durations before imaging. All ΔFBS samples were diluted to 0.5% v/v in a C\text{pH} = 6.5 phosphate buffer with 130 mM NaCl. Imaging solution in a C\text{pH} = 6.0 phosphate buffer containing 50 μM L-012, 200 μM H₂O₂, 20 mM NaBr, and 500 mM NaCl was added to the plate after multiple washes removed any non-adsorbed ΔFBS and MPO. Raw data (n = 3) are shown. (B) WLS regression on data from the 5 min timepoint of (A). Triplicate 95% prediction bands are shown as long-dashed lines outside the regression mean and shaded 95% confidence bands. Data are shown as mean ± standard deviation.
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**Figure 3.7: Anticoagulant heparin blocking MPO adsorption.** (A) Bioluminescence time series of human plasma drawn with different anticoagulants, supplemented with purified human MPO, and adsorbed onto polystyrene. Plasma ± MPO was diluted to 1\% v/v in DPBS and incubated for 30 min before the plate was washed free of unadsorbed material and imaged with 200 µM H₂O₂, 50 µM L-012, and 20 mM NaBr in an acidic phosphate buffer. MPO was unresolvable in the heparinized plasma. Each line is an individual well (n = 4). (B) Data from the 5 min timepoint analyzed with WLS regression. Mean ± standard deviation are shown. Long-dashed lines are triplicate 95% prediction intervals around the WLS mean (dashed line) and 95% confidence bands (gray). Heparinized plasma could not be fit with the regression method.

WLS regression. (Figure 3.7B) Blood anticoagulated with citrate also allowed for MPO adsorption, but K₂EDTA was exclusively used for consistency. (Data not shown)

Although MPO reliably adsorbed in a near-linear manner when incubated with either ΔFBS or human plasma on tissue culture-treated polystyrene, the resulting bioluminescence was not reproducibly equivalent with the two carriers. Generally, ΔFBS resulted in greater signal from equivalent MPO in human plasma. (Figure 3.8A) Modifying sample preparation techniques counteracted this discrepancy, but the results of this strategy were extremely sensitive to slight variations in the duration and extent of pipette transfers, sequence of sample dilutions, and even the type of liquid vessel used to mix each solution. (Data not shown) MPO in human plasma was linearly underestimated when measured using ΔFBS-based standards for WLS regression. (Figure 3.8B)
Figure 3.8: ΔFBS and human plasma as MPO adsorption carriers. (A) Bioluminescence of MPO adsorbed onto polystyrene in the presence of ΔFBS or human plasma. Data from the 5 min timepoint are shown as mean ± standard deviation (n = 4). WLS regression mean ± 95% confidence bands are shown as dashed lines and gray areas, respectively. Quadruplicate prediction intervals are shown in long-dashed lines. MPO is annotated as pre-dilution concentrations in each carrier tissue. (B) WLS regression results of MPO in human plasma calibrated against MPO standards in ΔFBS. Data are shown as mean ± 95% confidence interval. Dashed line represents 1:1 agreement between supplemented and regressed MPO.

Anticoagulated human plasma and ΔFBS differ greatly in protein content as measured by the bicinchoninic acid protein assay (data not shown) as would be expected after the removal of clotting factors, fibrinogen, and co-aggregated proteins during the plasma-serum conversion. Additionally, ΔFBS is heat-treated with the intention of denaturing serum proteins, further differentiating it from plasma. From this, RBP was tested as an alternative carrier to ΔFBS for assaying human MPO. As seen with human plasma, RBP co-adsorbed with exogenous human MPO with reproducible signal and kinetics. (Figure 3.9A) Calibration curves prepared from MPO in RBP were linear or slightly convex and had narrow prediction intervals, both desirable traits in an assay standard. (Figure 3.9B) Most importantly, human plasma supplemented with exogenous MPO were reliably assayed against RBP-based standards; the WLS regression results agreed within 95% confidence limits with supplemented MPO.
Figure 3.9: RBP and human plasma as MPO adsorption carriers. (A) Time series of human MPO serially diluted into human plasma or RBP before dilution to 0.4% v/v and incubating for 30 min in a polystyrene microtiter plate. Unadsorbed material was washed from the plate before imaging with 100 µM H$_2$O$_2$, 50 µM L-012, and 200 mM (NH$_4$)$_2$SO$_4$ in an acidic citrate buffer. Individual wells (n = 4) are shown. (B) WLS calibration curve of data from the RBP solutions at the 5 min timepoint. This standard curve had a limit of detection = 1.8 µg/L MPO with $R^2 > 0.998$. Data are shown as mean ± standard deviation. Quadruplicate 95% prediction bands are shown as long-dashed lines about the dashed WLS mean and gray 95% confidence bands. (C) Human plasma calibrated against the RBP curve. Solutions were accurately predicted within experimental error. Dashed line represents perfect 1:1 agreement between supplemented MPO and inverse regression results. Data are shown as regression estimate ± 95% confidence interval.

concentrations in plasma from a healthy donor. (Figure 3.9C) The utility of RBP as a human plasma substitute was confirmed with two product lots obtained independently. (Data not shown)

RBP was roughly fractionated by membrane ultrafiltration with 5, 30, and 100 kDa exclusions to determine if specific proteins were necessary or sufficient for MPO adsorption.
Figure 3.10: Plasma size fractionation and MPO adsorption. RBP was fractionated by centrifugal ultrafiltration before both the filtrate and retained feed from each size-cutoff was used as a carrier solution for MPO adsorption. Each filtrate was diluted to 0.5% v/v in a pH = 6.5 phosphate buffer and incubated in a polystyrene microtiter plate for 30 min. After washing unadsorbed material from the plate, it was imaged with 100 µM H₂O₂, 50 µM L-012, 20 mM NaBr, and 200 mM (NH₄)₂SO₄. Data from the 5 min timepoint are shown as mean ± standard deviation (n = 4). WLS results are shown as dashed lines ± gray 95% confidence bands. Quadruplicate 95% prediction bands are shown as long-dashed lines. MPO axis represents pre-dilution concentration.

Of the retained feeder plasma components, only the >100 kDa cutoff showed slightly reduced bioluminescence. The matched <100 kDa filtrate likewise allowed MPO adsorption above that of buffer or the smaller molecular weight cutoff filtrates. Although the >5 and >30 kDa fractions showed slightly increased signal compared to that of whole RBP control, it is uncertain if this resulted from the loss of the <5 kDa fraction or if it was an artifact of differences in sample dilution due to the uncertain retention volume of the centrifugal filter units. (Figure 3.10)

A homogeneous and consistent MPO carrier is desirable to reduce the number of possible confounding assay components. While both human plasma and RBP evenly coated the floor surface of microtiter plate wells, there are many unknown parameters in such complex protein mixtures. (Figure 3.11A) Plasma proteins known to bind to MPO were thus tested as pure co-adsorption additives. BSA has been shown to bind to human MPO and adsorb onto polystyrene surfaces. If sufficient to cause MPO adsorption, BSA would be an inexpensive...
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#### Results

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**Figure 3.11: MPO co-adsorption with pure plasma proteins.** (A) Tissue culture-treated polystyrene wells imaged after MPO adsorbed in the presence of 0.4% v/v human plasma and RBP. Images are 1 min integrations taken 5 min after the addition of the imaging solution containing 100 µM H₂O₂, 50 µM L-012, 20 mM NaBr and 200 mM (NH₄)₂SO₄. The even distribution of signal across the well-bottom surface is typical of MPO coadsorbed with whole plasma after citrate or EDTA anticoagulation. MPO is labeled as pre-dilution concentration in whole plasmas. (B) Wells imaged with pure BSA as an MPO adsorption carrier. MPO solutions were diluted to 0.2% v/v before incubation in a similar manner as used in (A). Signal rings around the well periphery is typical of residual solution desiccating during the washing steps, precipitating MPO and other proteins as opposed to uniform adsorption onto the polystyrene surface. MPO is annotated with pre-dilution concentrations, BSA with final incubation concentrations. (C) Wells imaged with pure human serum fibronectin as an MPO adsorption carrier. MPO solutions were diluted to 0.4% v/v before incubation in a similar manner as used in (A) and (B). As in (B), ringing indicates poor adsorption compared to that from MPO + plasma mixtures. MPO is annotated with pre-dilution concentrations, fibronectin with final incubation concentrations.

and consistent assay component. However, MPO and BSA proved insufficient to evenly coat wells, with signal rings typical of drying artifacts during plate washes composing most of the measurable signal. (Figure 3.11B) Fibronectin has also been shown to bind MPO and is known to adsorb readily onto modified polystyrene.[34,36–38] Fibronectin exists freely in plasma as well as in the extracellular matrix and, unlike BSA, is far larger than 100 kDa and compatible with the size fractionation data shown in Figure 3.10.[39] Although purified human fibronectin did cause increased adsorbed MPO bioluminescence more than observed
with BSA, even at high concentrations it was still insufficient to produce the even, consistent adsorption seen with whole human plasma or RBP. (Figure 3.11C)

### 3.3.4 MPO adsorption across solution conditions

There are no reports of MPO adsorption conditions in the literature, so a small screen was performed to coarsely investigate several solution and processing parameters for guidance in further assay optimization. Incubation buffer parameters included pH, buffering agent, and NaCl concentration. Based on the observed discrepancy between ΔFBS and RBP (see Figure 3.8 and Figure 3.9), attempts to selectively remove plasma proteins other than MPO and those required for its adsorption were made. Pre-incubation and centrifugation with varying CaCl\(_2\) was explored to saturate the anticoagulant K\(_2\)EDTA and remove polymerized fibrin and associated proteins prior to polystyrene incubation. Pre-incubation and centrifugation with concentrated (NH\(_4\))\(_2\)SO\(_4\) was also investigated, as (NH\(_4\))\(_2\)SO\(_4\) is known to precipitate many abundant plasma proteins while MPO remains soluble.\(^{40-42}\) Treatment and solution conditions are summarized in Table 3.1.

Although precipitate pellets were visible after centrifugation with (NH\(_4\))\(_2\)SO\(_4\), it had no beneficial effect on MPO adsorption from the supernatants. Of note, each of the supernatants still contained 10% of the centrifuge (NH\(_4\))\(_2\)SO\(_4\) concentration after dilution into the plate incubation buffer. This was an unavoidable consequence of the precipitation step and must be interpreted as part of each treatment. (Figure 3.12A) Similarly, CaCl\(_2\) pretreatment consistently had no effect on bioluminescence of adsorbed MPO despite a visible pellet present after centrifugation. (Figure 3.12B) Further experimentation with plasma fractionation via selective precipitation with (NH\(_4\))\(_2\)SO\(_4\), CaCl\(_2\), and purified bovine thrombin similarly produced only negative results. (Data not shown) Of the conditions without a precipitation
Table 3.1: Multi-parameter survey of treatments for MPO adsorption. Human plasma ± exogenous human MPO was diluted to 10% v/v into each of the 16 test solutions. Preceding experiments suggested that pH, buffering agent, buffer concentration, coagulation cascade activation, and ionic strength had potentially important effects on MPO adsorption onto tissue culture-treated polystyrene. Blocks of conditions designed to test a particular parameter are shown. Conditions 5–15 were designed to precipitate plasma proteins such as fibrin(ogen) and thus were subjected to centrifugation; the resulting supernatant was transferred at the appropriate dilution into a matched loading solution shown in parentheses. Control solutions used in multiple comparison blocks are denoted by bold condition labels. Data from this adsorption screen are shown in Figure 3.12.

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step, only pH = 6.5 with 150 mM NaCl resulted in a substantial improvement in both absolute and relative difference between native plasma and plasma + 100 µg/L MPO. (Figure 3.12C) The signal ratios of each condition corresponding with Table 3.1 are shown in Figure 3.12D.

From this survey, pH and NaCl concentration stood out as important adsorption solution parameters. To find the optimum pH for adsorbed MPO retention, a ratio titration of mixed NaH_2PO_4 and Na_2HPO_4 totaling 25 mM was performed in the presence of 130 mM NaCl. pH strongly influenced adsorption with optimal bioluminescence at \(^\text{C}\)pH = 6.6, agreeing
Figure 3.12: Multi-parameter survey of treatments for MPO adsorption. All data represent the 1 min image taken at the 5 min imaging timepoint (n = 3). Solution numbers are also annotated on each horizontal axis. (A) Effects of (NH₄)₂SO₄ pre-incubation and centrifugation in three buffer conditions before further diluting the supernatant into the matching 0 mM (NH₄)₂SO₄ buffer for adsorption. Although some precipitate remained after centrifugation with >1 M (NH₄)₂SO₄, pretreatment did not improve MPO resolution in any of the buffer conditions. (B) Varying CaCl₂ to reverse K₂EDTA anticoagulation from the phlebotomy tubes. Some visible precipitate remained after centrifugation, but there was little improvement over the Ca²⁺ control solution (#3). (C) Citrate buffer with a pH = 6.0 or phosphate buffers with pH = 6.5 or 7.0 with 0 or 150 mM NaCl. These conditions did not involve centrifugation and were kept at 4 °C while other solutions were centrifuged so that they could serve as controls when applicable. The effects of pH are discussed further in Figure 3.13. Of the different pH and NaCl permutations, only 150 mM NaCl at pH = 6.5 stood out with improved MPO resolution. (D) The signal ratio of MPO-supplemented plasma to untreated plasma after each of the 16 adsorption conditions. Ratios were determined by log-linked gamma regression and are displayed as mean ± 95% confidence intervals. For solution conditions included in this survey, see Table 3.1.
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**Figure 3.13: Optimal pH for MPO adsorption.** RBP with varying MPO was diluted to 0.4% v/v into phosphate buffers with 130 mM NaCl and different pHs (total PO$_4^{3-}$ = 25 mM). Wells were washed with the same phosphate buffer after plasma adsorbed over a 30 min incubation. The plate was imaged with 50 µM L-012, 100 µM H$_2$O$_2$, 200 mM (NH$_4$)$_2$SO$_4$, and 20 mM NaBr. (A) Data from the 5 min timepoint are shown as mean ± standard deviation (n = 4). WLS results are shown as dashed lines ± gray 95% confidence bands. Quadruplicate 95% prediction bands are shown as long-dashed lines. MPO axis represents pre-dilution concentration. (B) Data arranged to show bioluminescence across all tested pH values. Raw bioluminescence measurements shown.

with the previous data from the condition survey. (Figure 3.13) Further pH testing showed dramatic decreases in adsorbed MPO activity in citrate buffers with pH < 6.0 and at pH > 8 buffered by CAPS. (Data not shown)

### 3.3.5 Optimizing adsorbed MPO-dependent bioluminescence

Imaging MPO-dependent bioluminescence with L-012 requires optimization beyond simply maximizing MPO halogenation activity; releasing adsorbed MPO from the polystyrene surface must also be considered, as must the elimination of any inhibition activity present in co-adsorbed proteins from plasma. Relevant parameters were first surveyed similarly to what is described on page 106: pH, NaCl concentration, (NH$_4$)$_2$SO$_4$ concentration with Na$_2$SO$_4$ as a control for NH$_4^+$, and total buffer phosphate. Chance observations from an attempt to precipitate plasma proteins suggested that (NH$_4$)$_2$SO$_4$ mitigated some of the MPO
Table 3.2: Multi-parameter survey of adsorbed MPO imaging solutions. Human plasma ± exogenous human MPO was diluted to 1% v/v and incubated for 30 min on tissue culture-treated polystyrene, washed, and imaged with 16 solutions designed to survey different parameter effects on MPO resolution. Each solution also contained 20 mM NaBr, 50 μM L-012, and 100 μM H₂O₂. Preceding experiments suggested that phosphate buffer concentration, pH, ionic strength, and NH₄⁺ affected resolution of adsorbed MPO. Blocks of conditions designed to test a particular parameter are shown. Control solutions used in multiple comparison blocks are denoted by bold condition labels. Data from this imaging solution screen are shown in Figure 3.14.

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Bioluminescence inhibition caused by plasma components such as ceruloplasmin. Additionally, previous literature suggested that NaCl added to increase ionic strength could disrupt the interaction between MPO and ceruloplasmin.²⁵ Because previous data had shown Cl⁻ to inhibit HOBr-dependent MPO bioluminescence (see Figure 2.6 on page 55), varying concentrations of buffer phosphate were also used to independently adjust ionic strength. Preliminary data had also suggested that higher-capacity buffers prevented diluted plasma from increasing the imaging solution pH and minimizing MPO-independent L-012 oxidation. (Data not shown)

The imaging solution parameters tested are detailed in Table 3.2.
Of the parameters tested, \((\text{NH}_4)_2\text{SO}_4\) had the most profound effect. Bioluminescence from native plasma decreased with increasing \((\text{NH}_4)_2\text{SO}_4\). The same was true with MPO-supplemented plasma imaged with pH = 7 buffer; this was likely due to its effect on pH. For example, measurements with an electronic pH meter showed that 200 mM \((\text{NH}_4)_2\text{SO}_4\) lowered the pH of 25 mM phosphate buffer from 6.0 to 5.5. Despite the overall decrease in signal at buffer pH = 7, the absolute and relative difference between MPO-supplemented and native plasma increased with \((\text{NH}_4)_2\text{SO}_4\) concentration in both buffers. Additionally, absolute MPO-dependent bioluminescence in acidic phosphate buffer increased with \((\text{NH}_4)_2\text{SO}_4\). (Figure 3.14A) This boost in MPO resolution was greater than the improvement due to increasing NaCl. (Figure 3.14B) While total phosphate concentration was correlated with overall bioluminescence intensity, MPO resolution did not improve with increasing phosphate alone. (Figure 3.14C) The signal ratios of each condition corresponding with Table 3.2 are shown in Figure 3.14D.

A higher-resolution titration of \((\text{NH}_4)_2\text{SO}_4\) in the imaging solution was performed to characterize its effect. When applied to MPO-supplemented human plasma adsorbed onto polystyrene, \((\text{NH}_4)_2\text{SO}_4\) enhanced bioluminescence with a sigmoid relationship described by equation (3.2). This gave an \(EC_{50} = 34\) mM (95% confidence interval 32–36 mM) and a Hill coefficient \(H = 2.3\) (95% confidence interval 2.0–2.5) at all levels of supplemented MPO. Supplemented plasma showed an \(-10\)-fold asymptotic increase in bioluminescence intensity. (Figure 3.15A) When a similar experiment was performed with MPO-supplemented plasma diluted directly into imaging buffer, \((\text{NH}_4)_2\text{SO}_4\) did not cause a quantifiably sigmoid response curve. Analysis with equation (3.3) showed that although \((\text{NH}_4)_2\text{SO}_4\) in plasma gave a similar \(EC_{50}\) to that seen with adsorbed MPO, 23 mM (95% confidence interval 11–35 mM), the asymptotic maximum was <2-fold higher than baseline. (Figure 3.15B) The enhancing effect
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Figure 3.14: Multi-parameter survey of adsorbed MPO imaging solutions. All data represent the 1 min image taken at the 5 min imaging timepoint (n = 3). Solution numbers are also annotated on each horizontal axis. (A) Titration of (NH₄)₂SO₄ at pH = 6.0 or 7.0. The effect trend was the same in both buffer systems: (NH₄)₂SO₄ substantially increased overall MPO resolution. Decreased total luminescence in the pH = 7.0 buffer may be due to the pH shift from NH₄⁺. The effects of (NH₄)₂SO₄ are discussed further in Figure 3.15. (B) NaCl concentration at two pHs, 6.0 and 7.0. At high NaCl concentrations, the MPO inhibitor ceruloplasmin has been reported to dissociate from MPO. Previous data also demonstrates that Cl⁻ inhibits Br⁻-dependent MPO bioluminescence at concentrations adequate to dissociate MPO and ceruloplasmin. (see Figure 2.6) As expected from this, increasing Cl⁻ decreases total luminescence at both pHs but slightly improves MPO resolution. (C) Total phosphate concentration at two pHs, 6.0 and 7.0. In addition to improved buffering capacity, increasing the total phosphate concentration also has a large effect on ionic strength. Increasing phosphate increased the overall luminescence, but did not have a substantial effect on MPO resolution. (D) The signal ratio of MPO-supplemented plasma to untreated plasma after each of the 16 imaging conditions. Ratios were determined by log-linked gamma regression and are displayed as mean ± 95% confidence intervals. For solution conditions included in this survey, see Table 3.2.
of \((\text{NH}_4)_2\text{SO}_4\) was absent when MPO was diluted into imaging solution in the absence of human plasma. (Figure 3.15C) To determine if the key solute was either \(\text{NH}_4^+\) or \(\text{SO}_4^{2-}\) individually, \(\text{Na}_2\text{SO}_4\) was titrated in imaging solution into which human plasma with varying supplemented MPO were added. Unlike the mild enhancement seen with \((\text{NH}_4)_2\text{SO}_4\) in Figure 3.15B, \(\text{Na}_2\text{SO}_4\) did not increase MPO bioluminescence. (Figure 3.15D)

The different effects of \((\text{NH}_4)_2\text{SO}_4\) when used with adsorbed plasma versus plasma diluted into buffer could be explained by the presence or absence of antioxidants, the removal of which was the rationale behind the development of the solid-state MPO assay. To test the removal of small molecule antioxidants, the potent bioluminescence inhibitor ascorbic acid (see Figure 2.12 on page 63) was added to RBP with varying human MPO before adsorption onto polystyrene. After washing unadsorbed material free from the plate, ascorbic acid did not show the potent inhibitory effect seen with MPO in solution. (Figure 3.16)

Tween20 was also discovered to have a profound effect on adsorbed MPO-dependent bioluminescence at multiple stages in the assay protocol. When added to pure MPO in solution, Tween20 did not affect MPO-dependent bioluminescence. (Data not shown) When added to the imaging solution applied to MPO adsorbed in conjunction with RBP, Tween20 caused a slight increase in bioluminescence at a final concentration \(\sim100\) ppm \(v/v\). (Figure 3.17) An \(\sim2\)-fold increase was observed when Tween20 was added at 65–130 ppm \(v/v\) to the RBP dilution and incubation buffer. (Figure 3.18) Most striking by far was the improvement seen by 200 ppm \(v/v\) Tween20 in the MPO standard curve dilution buffer, which not only increased the signal by a factor \(>4\), but also dramatically reduced the intra- and inter-assay variance of prepared standard curves. (Figure 3.19)
Figure 3.15: Increasing adsorbed plasma MPO activity with (NH₄)₂SO₄. Data are from the 5 min timepoint and are shown as mean ± standard deviation (n = 4). (A) Titration curves of (NH₄)₂SO₄ in imaging solutions for adsorbed MPO. (NH₄)₂SO₄ was titrated in the presence of 50 µM L-012, 100 µM H₂O₂, 20 mM NaBr, and 200 ppm v/v Tween20 in an acidic citrate buffer. MPO concentrations are in whole plasma before dilution to 0.4% v/v in loading buffer for adsorption onto polystyrene. Regression results ± 95% confidence bands from equation (3.2) are shown as dashed lines and gray areas, respectively. (NH₄)₂SO₄ enhanced MPO bioluminescence with EC₅₀ = 34 mM (95% confidence interval 32–36 mM). The regression Hill coefficient and EC₅₀ did not vary significantly between the three MPO concentrations.

(B) Titration curves of (NH₄)₂SO₄ in imaging solutions for MPO in human plasma. (NH₄)₂SO₄ was titrated in the presence of 50 µM L-012, 100 µM H₂O₂, 20 mM NaBr in an acidic citrate buffer. MPO concentrations are in whole plasma before dilution to 400 ppm v/v in imaging buffer. Regression results ± 95% confidence bands from equation (3.3) are shown as dashed lines and gray areas, respectively. (NH₄)₂SO₄ enhanced MPO bioluminescence in the 300 µg/L and 100 µg/L solutions with EC₅₀ = 23 mM (95% confidence interval 11–35 mM). The regression EC₅₀ did not vary significantly between the two MPO concentrations that could be fit to equation (3.3). (C) Titration curves of (NH₄)₂SO₄ in imaging solutions for pure MPO in acidic citrate buffer with 20 mM NaBr, 30 µM L-012, and 100 µM H₂O₂. Unlike in (A) and (C), (NH₄)₂SO₄ did not substantially enhance MPO bioluminescence. (D) Modification of the experiment used in (B) using Na₂SO₄ in place of (NH₄)₂SO₄ to control for ionic strength and SO₄²⁻ concentration. Na₂SO₄ did not enhance MPO bioluminescence and could not be fit to standard models of either enhancement or inhibition. MPO concentrations are in whole plasma before dilution to 400 ppm v/v in imaging buffer.
Figure 3.16: Ascorbic acid washed from adsorbed MPO. Bioluminescence from RBP supplemented with human MPO and varying ascorbic acid prior to adsorption and imaging on polystyrene. Black bar represents the physiological reference range for plasma ascorbic acid. Unlike when applied directly to MPO in imaging solution, ascorbic acid has no substantial effect on adsorbed MPO bioluminescence. Data from the 5 min imaging timepoint are shown as mean ± standard deviation (n = 4). MPO and ascorbic acid are annotated with pre-dilution concentrations. Physiologic reference range from McPherson & Pincus.43

Figure 3.17: Effects of Tween20 in bioluminescence imaging buffer applied to adsorbed MPO. MPO co-adsorbed with RBP after dilution to 0.4% v/v in 0.05M pH = 6.5 phosphate buffer and imaged with 100 µM H₂O₂, 50 µM L-012, 200 mM (NH₄)₂SO₄, 20 mM NaBr, and varying Tween20. Tween20 had a minimal effect on MPO resolution with peak improvement at ~50–140 ppm v/v. All data are from the 5 min timepoint of their respective time series and are shown as mean ± standard deviation (n = 4). WLS results are shown as dashed lines ± gray 95% confidence bands. Quadruplicate 95% prediction bands are shown as long-dashed lines. MPO axis represents pre-dilution concentration.
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Figure 3.18: Improved adsorption of MPO in the presence of Tween20. Modification of the experiment used in Figure 3.17 using Tween20 in the loading buffer into which RBP ± MPO was diluted to 0.4% v/v and incubated on the polystyrene plate. ~29–65 ppm v/v Tween20 resulted in the greatest improvement in MPO resolution. All data are from the 5 min timepoint of their respective time series and are shown as mean ± standard deviation (n = 4). WLS results are shown as dashed lines ± gray 95% confidence bands. Quadruplicate 95% prediction bands are shown as long-dashed lines. MPO axis represents pre-dilution concentration.

Figure 3.19: Tween20 reducing MPO loss during serial dilutions. Modification of the experiment used in Figure 3.17 and Figure 3.18 using Tween20 in the MPO serial dilution buffer. Using this method, pure MPO was combined with pre-diluted RBP rather than premixing the two before dilution. MPO resolution improved dramatically and plateaued by 200 ppm v/v Tween20. All data are from the 5 min timepoint of their respective time series and are shown as mean ± standard deviation (n = 4). WLS results are shown as dashed lines ± gray 95% confidence bands. Quadruplicate 95% prediction bands are shown as long-dashed lines. MPO axis represents pre-dilution concentration.
3.3.6 Bioluminescence specificity for adsorbed MPO

The specific MPO inhibitor 4-ABH was used to verify that the bioluminescence from adsorbed plasma supplemented with exogenous MPO was from active enzyme.\(^{44}\) Titrating 4-ABH into the imaging solution applied to adsorbed RBP with human plasma inhibited bioluminescence fitting the model described by equation (3.4) with an \( IC_{50} = 25 \text{ nM} \) (95% confidence interval 22–28 nM). This also provided further evidence that RBP is a suitable baseline carrier for an MPO assay: native RBP bioluminescence cannot be fit to equation (3.4) and remained at a steady baseline across the entire titration concentration range. (Figure 3.20A) To further test that MPO was adsorbed onto the polystyrene surface, the highly cationic protein protamine sulfate was co-incubated at increasing concentrations with RBP supplemented with human MPO. Protamine sulfate blocked MPO adsorption with an \( IC_{50} = 590 \mu\text{g/L} \) (95% confidence interval 560–620 μg/L). (Figure 3.20B) This effect was confirmed to be caused by competitive adsorption by testing protamine sulfate for intrinsic inhibition activity against MPO bioluminescence. Even when applied at concentrations over many orders of magnitude, protamine sulfate had no effect on MPO bioluminescence in solution. (Figure 3.20C)

3.4 Discussion

Plasma MPO content is a recently discovered biomarker for subclinical CVD progression, risk of MACEs in patients with known coronary artery disease (CAD), and short- and long-term prognosis after clinical presentation with a MACE.\(^{9–20}\) At present, plasma MPO is measured with ELISAs, which are costly, time-consuming, and do not utilize the enzymatic properties of MPO in its detection. Luminol and its analogue L-012 serve as bioluminescence substrates for MPO activity by means of chemiluminescent oxidation caused by hypohalous acids produced
Figure 3.20: Bioluminescence specificity for adsorbed MPO. All MPO is annotated with predilution concentrations. (A) Titration of the specific MPO inhibitor 4-ABH in imaging solutions applied to MPO adsorbed in the presence of RBP. Regression of the data from MPO-supplemented RBP with equation (3.4) showed that 4-ABH inhibited bioluminescence with an $IC_{50} = 25$ nM (95% confidence interval 22–28 nM). Native RBP did not fit the inhibition model and signal remained at the baseline across all 4-ABH concentrations. Data from the 5 min imaging timepoint are shown as mean ± standard deviation ($n = 4$). Nonlinear regression and 95% confidence bands are shown as dashed lines and shaded areas, respectively. (B) Titration curves of protamine sulfate co-incubated with RBP supplemented with varying MPO and diluted to 0.4% $v/v$. Protamine sulfate is a cationic protein much like MPO and can compete for adsorption surface area. Regression with equation (3.5) gave an $IC_{50} = 590$ µg/L (95% confidence interval 560–620 µg/L). Data from the 5 min imaging timepoint are shown as mean ± standard deviation ($n = 4$). Nonlinear regression and 95% confidence bands are shown as dashed lines and shaded areas, respectively. (C) Protamine sulfate titrated with human plasma supplemented with exogenous MPO and diluted to 500 ppm $v/v$. Unlike with bioluminescence from adsorbed MPO seen in (B), protamine sulfate does not affect MPO bioluminescence in solution. Raw data from the 5 min imaging timepoint are shown ($n = 4$).

As shown in Chapter 2, assaying MPO activity with L-012 and $\text{H}_2\text{O}_2$ added directly to plasma is not a feasible strategy; nonspecific antioxidants and endogenous MPO inhibitors are confounding variables with non-linear relationships to MPO bioluminescence and can vary widely between samples. This chapter describes the inception and optimization of a novel bioluminescence assay for MPO activity from whole human plasma. The objectives in developing the assay were to measure human MPO activity with similar precision and reproducibility compared to current commercial ELISA kits but with great reductions in monetary cost, required time, and procedural steps.
Chapter 3: Myeloperoxidase sequestration on solid substrates

Discussion

Enzyme peroxidase activity is commonly measured on various polymer membrane surfaces, often to visualize protein bands with immunoconjugated HRP targeting a desired epitope. The ubiquity, low cost, and simplicity of protein isolation on nitrocellulose or PVDF membranes made the technique an intuitive platform to utilize for MPO retention. As expected from the hydrophilic oligosaccharides adorning its surface and strong cationic charge of the core protein, MPO was retained after direct application to nitrocellulose with concentration-dependent bioluminescence visible after buffer washes. (Figure 3.1A) Such dot-blot techniques are generally used when the analyte concentration spans orders of magnitude or when precise quantification is unnecessary, as is not the case with plasma MPO. With an improvised apparatus for applying solutions to a nitrocellulose membrane, the distribution of bioluminescence from each sample became more heterogeneous with sharp boundaries for improved quantification. (Figures 3.1B–3.1D)

The functionality of the nitrocellulose cassette proof-of-concept is similar to commercially available protein analysis products, which have the advantage of consistency and quality control far exceeding that of re-purposed laboratory plastic waste. PVDF-floored microtiter plates did not produce linear bioluminescence with either luminol or L-012, likely due to decreased affinity for hydrophilic macromolecules compared to that of nitrocellulose. (Figure 3.2) Contrary to expectations, nitrocellulose-floored plates also performed poorly when used to distinguish MPO from a negative control solution with L-012 bioluminescence imaging, both in the presence and absence of diluted human plasma. (Figure 3.3A) As shown in Figure 2.9B on page 60, acidification of the imaging solution improves the signal ratio of MPO:H2O2-dependent L-012 oxidation. This strategy did not improve the utility of the nitrocellulose plate in measuring MPO; although background signal decreased as expected, the same was equally true of MPO bioluminescence. (Figure 3.3B) Unlike the free
nitrocellulose membranes previously used successfully, the undersides of the filter plates were covered in a hydrophobic film intended to assist in vacuum filtration of fluid through each well. Although vacuum filtration through the plates required a manifold built to the plate specifications, a similar product allowed for vacuum filtration through free nitrocellulose membranes. Using atmospheric pressure to force MPO-laden solutions through the membrane greatly improved bioluminescence detection compared to that with passive plate loading, but this also revealed new disadvantages. Bioluminescence kinetics were extremely variable, even with pure solutions of MPO in buffer. (Figure 3.4A and 3.4B) Rigorous washing of the cassette before imaging mitigated this somewhat, especially at lower concentrations of MPO and allowed progression to testing with MPO diluted into ΔFBS. (Figure 3.4C) In the end, however, nitrocellulose imaging proved to have high MPO resolution in a narrow concentration range, with dilute MPO likely inhibited by other plasma components bound to the membrane, and higher concentrations saturating the available binding interface; the resulting sigmoid calibration curves were not amenable to inverse regression of unknown MPO solutions. (Figure 3.4D)

Plastics offer an alternative to polymer membranes for protein immobilization. They also have the advantages of precision manufacturing and chemical modifications that can be incorporated reproducibly into the well surfaces to increase protein binding. Sandwich ELISA plates are prepared in this manner by first incubating the desired immunoglobulin in buffers designed to maximize adsorption through non-covalent interactions at the protein/polymer interface. Initial attempts at coating various “high protein-binding” commercial microtiter plates with purified human MPO were discouraging; even conditions that resulted in near-linear relationships between loaded MPO concentration and measured bioluminescence had dynamic ranges unsuitable for a clinical assay. (Figure 3.5A) Dilute human plasma
supplemented with exogenous MPO paradoxically produced bioluminescence calibration curves with less variance than any solution-based MPO titrations (such as those in Chapter 2) and exceeding the quality of those from commercial ELISA kits. (Figure 3.5B) Tissue culture-treated polystyrene, while not marketed for this purpose, outperformed plastic surfaces designed for ELISA adsorption. (Data not shown) Additionally, MPO adsorption was complete in far less time than the 5–10 h or overnight incubations suggested by ELISA coating protocols. Although peak adsorption was achieved in 5–15 min, 30 min was chosen for all adsorptive incubations for consistency and to give adequate time to prepare any experimental imaging solutions during the optimization stages of assay development. (Figure 3.6)

Although human plasma from healthy volunteers was used extensively throughout the development of this assay, it has many disadvantages precluding its use as a standard carrier for MPO calibration solutions: it has greater associated biohazard risk than plasmas from other species, it is more costly to obtain, and by definition has the possibility of confounding endogenous human MPO. Another consideration in testing carrier plasmas was the means of anticoagulation. After a long series of failed experiments with fresh human plasma, the use heparinized phlebotomy tubes was found to prevent adsorption of any supplemented MPO. (Figure 3.7) This is agrees with the literature reports of MPO associating with heparin and other glycosaminoglycans. ΔFBS is another adsorption carrier used in assay development, but it often failed to accurately calibrate MPO added to human plasma. (Figure 3.8) The primary difference between human plasma and ΔFBS is the presence of fibrinogen and other proteins associated with thrombus formation. However, treating human plasma with bovine thrombin or CaCl₂ to initiate the fibrinogen → fibrin conversion had no effect on adsorbed MPO bioluminescence.
Reconstituted bovine plasma (RBP) anticoagulated with citrate reproducibly matched fresh human plasma anticoagulated with K$_2$EDTA as a human MPO adsorption carrier. (Figure 3.9) Calibration curves analyzed with WLS regression routinely produced $R^2 > 0.99$ and resulted in accurately regressed MPO adsorbed with human plasma within 95% confidence limits. RBP size fractionation was used to determine what plasma components are important to MPO adsorption. (Figure 3.10) Of the fractions above their respective molecular weight cutoffs that were tested, only the >100 kDa components showed slightly reduced co-adsorption utility. Removing the <5 kDa fraction appeared to increase MPO bioluminescence, but this may be an artifact of the filtration procedure, which could not be exactly replicated with unfiltered RBP, only approximated by incubating diluted RBP at 4 °C for the duration of the filtration spins. Still, BSA and human fibronectin were forerunner candidates for co-adsorption proteins given literature on both their interaction with MPO and adsorption onto activated polystyrene.$^{34-38}$ Neither proved sufficient to allow MPO adsorption, however, and after two independently obtained lots of RBP gave consistent results, efforts were shifted instead to optimizing the assay using whole RBP as the MPO carrier. (Figure 3.11)

Solution parameter surveys for both the adsorption and imaging solutions were performed to guide further optimization. Of the MPO binding treatments and solutions summarized in Table 3.1, those based on precipitating fibrin via addition of CaCl$_2$ to activate thrombin had the least effect on MPO bioluminescence. (Figure 3.12B) Additionally, all solutions with no NaCl performed poorly, suggesting that electrostatically driven protein aggregation at low ionic strength prevents MPO adsorption, but whether this is due to surface saturation by competing proteins or sequestration of MPO by other free proteins is not known. (Figure 3.12A) The most noteworthy comparison was between 150 mM NaCl buffered with citrate at pH = 6.0 and phosphate at pH = 6.5. (Figure 3.12C) The difference in buffer caused an ~2-fold
change in the signal ratio of MPO-supplemented plasma to native control, suggesting that protonation was the primary determinant of MPO adsorption. (Figure 3.12D) Titrating the ratio of NaH$_2$PO$_4$ to Na$_2$HPO$_4$ in a 25 mM phosphate buffer revealed the narrow pH optimum for MPO retention. (Figure 3.13) By chance, this maximum coincided to the pH used most often for plasma dilution in preceding experiments.

The most important finding made prior to testing the assay with clinical plasma samples involved abatement of co-adsorbed MPO inhibitors. The plasma protein ceruloplasmin is known to tightly bind to MPO and potently inhibit its activity \textit{in vivo} and \textit{in vitro} and also adsorbs onto oxidized polystyrene.$^{26,27,34,51,52}$ The interaction between MPO and ceruloplasmin can be disrupted by concentrated NaCl, but this is counterproductive to a bioluminescence assay optimized for bromination (see Figure 2.6 on page 55). As expected, increasing NaCl moderately improved adsorbed MPO bioluminescence at acidic and neutral pH. (Figure 3.14B)

An alternative salt to increase ionic strength is (NH$_4$)$_2$SO$_4$, which is commonly used by crystallographers and biochemists to “salt-out” proteins for purification or lattice formation due to its high solubility and the position of SO$_4^{2-}$ along the Hofmeister series.$^{53}$ Although one publication documented SO$_4^{2-}$ as an enhancer of luminol chemiluminescence, few published reports mention NH$_4^+$ in the context of MPO biochemistry, none of which investigate the interactions in a way directly applicable to bioluminescence.$^{1989a, 54–57}$ Interestingly, (NH$_4$)$_2$SO$_4$ has been reported as a potent inhibitor of HRP-dependent bioluminescence of luminol.$^{58}$ This made the discovery that (NH$_4$)$_2$SO$_4$ was a potent MPO bioluminescence enhancer rather unexpected. (Figure 3.14D) Acidification from NH$_4^+$ ions concentrated at 2–8 times the total buffering capacity accounts for the drop in overall luminescence in the C$pH = 7$ solutions, but this did not adversely affect MPO differentiation. Further investigation
of \((\text{NH}_4)_2\text{SO}_4\) enhancement after other optimization showed that \((\text{NH}_4)_2\text{SO}_4\) could increase MPO-dependent bioluminescence in the presence of co-adsorbed plasma proteins by a factor of \(\sim 10\) and the effect was sharply saturable. (Figure 3.15A) When titrated into diluted plasma supplemented with MPO, \((\text{NH}_4)_2\text{SO}_4\) enhancement was muted. (Figure 3.15B) This suggests that the antioxidants removed by the washes after MPO and other proteins adsorb to the plate are not affected by \((\text{NH}_4)_2\text{SO}_4\); its effect is on specific MPO inhibitors such as ceruloplasmin. From this, \((\text{NH}_4)_2\text{SO}_4\) would not be expected to enhance MPO bioluminescence in the absence of any inhibitors. This was confirmed experimentally. (Figure 3.15C) Additionally, the effect was dependent not on ionic strength of \(\text{SO}_4^{2-}\) concentration; titrating \(\text{Na}_2\text{SO}_4\) into diluted plasma supplemented with MPO showed no enhancement and actually reduced bioluminescence slightly. (Figure 3.15D)

Before applying the assay to clinical samples, it was first tested for specificity to MPO and robustness to small-molecule antioxidants. The potent inhibition observed from ascorbic acid titrated in the presence of pure MPO (see Figure 2.12A on page 63) was absent when RBP supplemented with human MPO and increasing ascorbic acid was incubated on polystyrene and washed free of unadsorbed material before imaging. (Figure 3.16) Even at the physiological reference range, ascorbic acid did not appreciably quench L-012 bioluminescence. The source of the bioluminescence was also determined to be functional MPO by two different lines of evidence. The specific MPO inhibitor 4-ABH quenched bioluminescence with an \(IC_{50} = 25\) nM (95% confidence interval 22–28 nM), consistent with pharmacological inhibition. (Figure 3.20A) The effect of co-incubation with a competing cationic protein also supports the conclusion that MPO is adsorbing from plasma onto the polystyrene; protamine sulfate blocked MPO adsorption with an \(IC_{50} = 590\) µg/L (95% confidence interval 560–620 µg/L) despite having no effect on MPO bioluminescence in solution. (Figure 3.20B and 3.20C) Both
of these experiments also confirm that RBP is a suitable standard MPO carrier, as native RBP did not fit the inhibition model with either experiment and remained at a constant baseline signal across both inhibitor titrations.

The final discovery important for the clinical assay was the effect of the detergent Tween20 on different stages of the protocol. Although RBP enabled accurate inverse regression of supplemented MPO in human plasma, both of these solution series were prepared by serially diluting MPO into the respective carriers. MPO was being lost at each serial dilution stage, but the effect was equal in the two series. As clinical plasma samples are not subject to this manipulation, early attempts to assay clinical samples were discordant from ELISA estimates of MPO concentration. (Data not shown) This MPO loss is apparent when Tween20 is added to the standard curve dilution buffer, resulting in a sharp rise in bioluminescence with Tween20 as dilute as 100 ppm v/v. (Figure 3.19) Additional adsorption is also apparent when Tween20 is added to the dilution buffer to which plasma is added before incubation in the microtiter plate wells. (Figure 3.18) Lastly, there is a slight increase bioluminescence from Tween20 in the imaging solution, perhaps preventing residual interactions between MPO and co-adsorbed inhibitors.

In conclusion, the work described in this chapter was aimed toward overcoming the obstacles to a clinical bioluminescence assay for plasma MPO activity described in Chapter 2. The primary achieved objectives were to isolate MPO from whole human plasma, remove or otherwise negate exogenous MPO inhibitors and antioxidants, and measure remaining MPO activity using calibration standards with known MPO activity. Nitrocellulose traditionally used for visualizing immunoconjugated HRP chemiluminescence limited the detectable concentration range of retained MPO due to high background signal, a saturable membrane interface, and retention of exogenous inhibitors. Although commercial plastic surfaces
intended to bind immunoglobulins for ELISA failed to linearly adsorb MPO, tissue culture-treated polystyrene proved apt for rapid MPO binding when incubated in the presence of human or bovine blood plasma. Human plasma MPO was accurately regressed using RBP standards with known MPO activity. Co-adsorbed inhibitors were neutralized with concentrated \((\text{NH}_4)_2\text{SO}_4\), increasing bioluminescence signal by an order of magnitude. The technique resulting from the work in this chapter, hereafter referred to as the ‘MPO activity on a polymer surface (MAPS) assay,’ produces calibration curves superior to those from ELISA at <5% of the cost and can be executed in 30 min. The next chapter will discuss a pilot clinical study on the accuracy and reproducibility of the MAPS assay in contrast to ELISA, the current clinical standard for measuring plasma MPO.

3.5 References


Chapter 4

Clinical ELISA and MAPS Assays

4.1 Introduction

Chapter 3 demonstrated the development and optimization of a novel method to measure myeloperoxidase (MPO) in plasma, dubbed the ‘MPO activity on a polymer surface (MAPS) assay.’ This research was motivated by the increasing need for cardiovascular disease (CVD) biomarkers, as the burden of the disease is rising both nationally and globally.1-3 Assessing the extent of CVD through plasma biomarkers is a desirable strategy as they can be measured with less risk and cost than invasive imaging techniques. Biomarkers of the inflammatory processes that drive atherosclerosis progression are a newer class under investigation to potentially join traditional analytes such as cardiac troponins.4-7 Plasma MPO concentration is a potentially useful biomarker in predicting CVD risk as indicated by a growing body of literature.8-30 Unlike the enzyme-linked immunosorbent assays (ELISAs) performed in these previous studies, the MAPS assay can detect a broad range of MPO in human plasma without the use of costly immunological reagents. It can also be performed in ~30 min, as opposed to the many hours required by ELISA protocols, and costs <5% of the per-sample ELISA cost. The MAPS assay has proven to be reproducible in simulated assays of clinical samples, but a trial with patient plasma was required to directly compare the results to
those from ELISA. In this chapter, MAPS assay data from a cohort of 72 cardiology patients presenting for elective central catheterization is compared to matched ELISA measurements. The two assays are compared with traditional methods of assay validation, including both a side-by-side ELISA and a Food and Drug Administration (FDA)-approved ELISA performed by a Clinical Laboratory Improvement Amendments (CLIA)- and College of American Pathologists (CAP)-accredited laboratory, the Cleveland HeartLab (CHL). Additionally, the MAPS assay is independently replicated four times with the entire cohort in order to quantify the inter-assay variance with a robust statistical method of quantifying reproducibility.

4.2 Methods

4.2.1 Reagents and materials

Lyophilized L-012 was provided by Wako Chemicals USA (Richmond, Virginia; product number 120-04891) was resuspended to 16.1 mM in ultrapure water and stored in 200 µL aliquots at −20 °C. Lyophilized human MPO from EMD Millipore/Calbiochem (Darmstadt, Germany; product number 475911) was resuspended in pH = 6.0 buffer containing 100 mM NaCl and 50 mM acetate, as recommended by the supplier, with final concentration 100 mg/L. Single-use 10 µL aliquots were stored at −20 °C. Lyophilized citrated bovine plasma from Sigma Aldrich (Saint Louis, Missouri; product number P4639) was resuspended to the original 10 mL with ultrapure water and gently agitated at room temperature until particulates had dispersed. Reconstituted bovine plasma (RBP) was stored at −20 °C. All other reagents were supplied by Sigma Aldrich. Human MPO ELISA kits were provided by Enzo Life Sciences, Inc. (Farmingdale, New York; product number ADI-900-115) All bioluminescence experiments were performed using 96-well, black-walled, flat-bottom, tissue culture-treated
polystyrene microtiter plates (Corning Inc.; Corning, New York; product number 3603). Samples were prepared for MAPS assays in 1.2 mL polypropylene cluster tubes (Corning Inc; product numbers 4401 and 4418).

4.2.2 Human plasma samples

Prior to elective cardiac catheterization, patients in the experimental cohort provided written and informed consent to allow collection of ~10 mL blood by a physician after successful arterial cannulation. Blood was transported on ice in K$_2$EDTA vacuum tubes for plasma isolation. This was accomplished by two-stage centrifugation at 4 °C: 1,000 × g in the collection tubes for 15 min followed by 14,000 × g in microcentrifuge tubes for 10 min. Plasma was stored at −80 °C and thawed at 4 °C before use. The plasma samples sent to CHL were stripped of any personal identifying information and assigned identifiers (name, date of birth, and sex) generated by an online name generator and random number generator.$^{31,32}$ Per the CHL protocol, samples were divided into aliquots with a minimum volume of 500 µL and shipped overnight at 4 °C; only 67 of the 72 samples met this volume requirement. The Institutional Review Board at Washington University in St. Louis approved these study protocols.

4.2.3 Bioluminescence imaging

All images were acquired with the ONYX/M imaging system with a XR/MEGA-10Z camera (Stanford Photonics; Palo Alto, California) at 55 frames/s with no optical filter. Images were integrated for 10 s to prevent camera saturation and compiled into 1 min exposures for quantification with the Fiji distribution of ImageJ analysis software.$^{33}$ All bioluminescence data are presented in dimensionless intensity units and are uniformly scaled throughout to
allow direct comparison between experiments. MAPS assay data were quantified at the 5 min
timepoint.

4.2.4 Data analysis and statistics

Bioluminescence data were quantified with the Fiji distribution of ImageJ image analysis
software. Images were processed to correct for optical vignetting as detailed in the Appendix
Section §7.4.2 on page 229. Regions of interest (ROIs) were created in microtiter plate arrays
using the Quadrangular ROI array program, the source code of which can be found on
page 226. Quantifications were normalized to image ROI pixel area to account for inter-
experiment differences in ROI layout. All statistical analyses were performed in IBM SPSS
Statistics 21 (Armonk, New York) unless otherwise noted.

4.2.5 WLS calibration of plasma MPO

Throughout this project, data were analyzed with a weighted least squares (WLS) regression
algorithm adapted from multiple literature reports on calibration methods. A detailed
description of the WLS calibration methods can be found in the Appendix Section §7.2 on
page 202. Briefly, the method constructs a standard curve by weighting each calibration
standard according to the inter-replicate variance and comparing first- and second-order
models on the basis of degrees of freedom and regression residuals. Assay data are usually
heteroscedastic with a positive correlation between signal and variance. When assays are
calibrated using ordinary least squares (OLS) regression, measurement heteroscedasticity
artificially increases the calculated uncertainty at the lower range of analyte standards.

\[^{1}\text{Often forgotten in biology literature, there is an intrinsic assumption with OLS regression that the data are homoscedastic. Although regression coefficients are not systematically biased by violating this assumption, estimates of dispersion such as the standard error of the mean are strongly biased. This is one of many reasons to take } p \text{-values lightly.}\]
It also increases overall variance by giving disproportionately equal weight to uncertain measurements. Alternatively, WLS regression gives more weight to measurement replicates with low variance with the assumption that deviation from the regression model in replicates with high variance is due to random processes. (Figure 4.1) The method used to analyze data from both assays is automated in regression model selection, weight assignments, and final prediction outputs to prevent any experimenter bias. For all comparisons in these experiments, WLS regression used technical triplicates ($m = 3$), 95% confidence intervals ($\alpha = 0.05$), and 95% power ($\beta = 0.05$).
4.2.6 Assay reproducibility metrics

The reproducibility of the MAPS assay was determined taking into consideration the uncertainty in each independent replicate. In place of traditional estimates of reproducibility, standard deviation $s$ and coefficient of variation $c_v$, variance-adjusted parameters $\textit{variability} \, \hat{s}$ and $\textit{coefficient of variability} \, \omega$ were used to quantify inter-assay dispersion. These parameters were calculated by adapting methods designed to combine variances from independent experiments for meta-analyses.$^{38,39}$ A detailed explanation of these calculations can be found in the Appendix Section §7.3 on page 223.

4.2.7 MPO ELISA

ELISAs on clinical samples were performed with slight modification to the protocol suggested by the manufacturer to provide data “in parallel” with MAPS. The standard curve dilution series was matched to that used in MAPS assays: 300, 225, 161, 107, 64.3, 32.1, 10.7, and 0 µg/L pre-dilution MPO. Plasma and MPO standard curve samples were loaded and analyzed in triplicate. All ELISA measurements were maintained as raw absorbance values throughout analysis. Additional ELISAs were performed by the CHL (Cleveland, OH), an independent CLIA- and CAP-accredited laboratory with an FDA-approved MPO assay. ELISA data from the CHL were requested as raw absorbance values and analyzed in the same manner. No modifications were requested to their processing techniques or calibration standards.
4.2.8 MAPS assay protocol

Two concentrated buffer\(^2\) stocks were used to make the three\(^3\) solutions required for the MAPS assay protocol: MAPS loading/wash buffer (PS-6.5), MPO dilution buffer (PS-6.5\(^T\)), and MAPS imaging solution (BLI-6). The components of these solutions can be found in Table 4.1. To minimize variance, the assay was performed with as little deviation from the protocol as possible.\(^4\)

At the beginning of each assay, plasma samples and RBP were removed from −80 °C storage and thawed on 4 °C cold-blocks or on ice. All pipetted sample preparation solutions were added to container walls with the reverse-volume method. For each sample to be assayed, a 1.2 mL polypropylene cluster tube was filled with 500 μL PS-6.5. A working MPO solution was made by adding 2 μL stock 100,000 μg/L MPO to 18 μL PS-6.5\(^T\) in a 200 μL polypropylene tube and mixing on a vortex mixer (working concentration: 10,000 μg/L). Eight additional 200 μL tubes each received PS-6.5\(^T\): 77.60 μL added to the first tube, 20 μL to each of the remaining seven. The first MPO standard was made by adding 2.40 μL 10,000 μg/L MPO to the 77.60 μL PS-6.5\(^T\) tube and mixing by vortex, final concentration 300 μg/L. From this, serial dilutions of 60, 50, 40, 30, 20, 10, and 0 μL were made from the preceding solutions into 20 μL PS-6.5\(^T\) with vortex mixing at each step. This produced a series of MPO standards with 300, 225, 161, 107, 64.3, 32.1, 10.7, and 0 μg/L MPO. 2 μL from these standards was added to separate cluster tubes already containing 500 μL PS-6.5. Plasma samples were then added as 2 μL volumes to the other prepared cluster tubes, which were sealed with polypropylene caps and mixed thoroughly\(^5\) on a vortex mixer. Finally, 2 μL

\(^2\)Stock solution pHs are calculated and are not adjusted prior to use. pH adjustment introduces solutes not tested in the assay and increases the frequency of solution contamination.

\(^3\)PS-6.5 and PS-6.5\(^T\) differ only by polysorbate 20 (Tween20) and shared a common concentrate.

\(^4\)The protocol is described in greater detail than in standard methods sections in the anticipation that this text will be used by others to replicate the assay in the future.

\(^5\)ensured by visually verifying that a sustained vortex formed in each tube.
RBP was added to each of the cluster tubes already containing pure MPO standard solutions, which were then mixed in the same manner as the plasma samples. Solutions were then loaded in triplicate 100 µL volumes to a tissue culture-treated polystyrene microtiter plate.\(^6\) Once loaded, the plate was covered and mixed for 30 min\(^7\) at 1,000 rpm on an orbital shaker.

The required volume of \textit{BLI-6} was prepared without L-012 or hydrogen peroxide (H\(_2\)O\(_2\)) during the incubation step and pipettes were set with the proper volumes for rapid addition of both reagents at the completion of the plate washes. On completion, the samples were transferred into a bleached vacuum flask with an 8-channel aspirator with suction applied to the rims of the well floors to remove as much liquid as possible without contacting the plastic surface. Each well was washed twice by addition of 150 µL \textit{PS-6.5} and aspiration as before to remove wash buffer. A third wash volume of 300 µL \textit{PS-6.5} was thoroughly aspirated from each well. L-012 and H\(_2\)O\(_2\) were added and rapidly mixed into \textit{BLI-6} before it was added in 150 µL volumes to each well. The plate was shaken briefly up to 1,000 rpm and imaged immediately in the ONYX/M. Data for quantification were obtained at the 5 min timepoint.

### 4.3 Results

#### 4.3.1 Patient characteristics

The clinical characteristics of the patient population as they presented prior to catheterization are shown in Table 4.2. Recruited patients had diverse demographic and clinical profiles. As expected, average plasma MPO concentration was lower in these patients presenting

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\(^6\) An 8-channel repeater pipette was used to deliver the samples. Tips were pre-wet with the full 300 µL volume and the exterior of the tips was touched to the cluster tube wall to remove any adherent liquid before the tips contacted the well walls.

\(^7\) The duration was chosen arbitrarily but used consistently and precisely. See Figure 3.6 on page 100 for the effects of incubation duration.
Table 4.1: MAPS assay solutions. Shorthand aliases (italics) are used throughout when discussing the MAPS protocol. Concentrated stocks were made in newly opened plastic labware to prevent contamination.

*200 mM \((\text{NH}_4)_2\text{SO}_4\) is estimated based on a saturated \((\text{NH}_4)_2\text{SO}_4\) solution calculated to be 4.05 M based on saturation data from the literature.\(^{40}\) These additives are not present in concentrated stocks; they are added immediately prior to use each assay.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Additive</th>
<th>Concentration</th>
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<tr>
<td>MPO dilution buffer</td>
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<tr>
<td>PS-6.5(^T)</td>
<td>(\text{NaH}_2\text{PO}_4)</td>
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<td></td>
<td>(\text{NaCl})</td>
<td>130 mM</td>
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<tr>
<td></td>
<td>Tween20(^t)</td>
<td>200 ppm (v/v)</td>
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<td>MAPS loading/wash buffer</td>
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<td></td>
<td>(\text{NaCl})</td>
<td>130 mM</td>
</tr>
<tr>
<td></td>
<td>Tween20(^t)</td>
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<tr>
<td>BLI-6</td>
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<td>3.09 mM</td>
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<td></td>
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<tr>
<td></td>
<td>((\text{NH}_4)_2\text{SO}_4)</td>
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</tr>
<tr>
<td></td>
<td>L-012(^t)</td>
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<tr>
<td></td>
<td>(\text{H}_2\text{O}_2)</td>
<td>100 (\mu)M</td>
</tr>
<tr>
<td></td>
<td>Tween20(^t)</td>
<td>100 ppm (v/v)</td>
</tr>
</tbody>
</table>

for elective catheterization than literature reports on higher-risk patients, such as those presenting with acute coronary syndrome (ACS). Indications of CVD progression were nonetheless elevated, noticeable in the prevalence of coronary artery bypass graft (CABG) (8), percutaneous coronary intervention (PCI) (21), and known coronary artery disease (CAD) (36). Of note, 17 patients had received intravenous heparin prior to sampling. As demonstrated in Chapter 3, heparin anticoagulation during phlebotomy eliminates MPO signal in the MAPS assay (see Figure 3.7 on page 101). Despite this possibility, samples from patients treated with heparin had detectable MAPS bioluminescence and were included in this analysis. Another possible confounder, thiocyanate, actually inhibits bromination as indicated by pure MPO bioluminescence. (Data not shown) Of the 72 patients, 39 reported that they are current smokers; literature reports on plasma SCN\(^-\) in active smokers indicate an ~2-fold increase over that of non-smokers.\(^{41,42}\)
Table 4.2: Clinical data from plasma donors. Mean ± standard deviation are given when applicable. Parentheses indicate absent patient data. Reported ancestry: Eur (European), Afr (African), Asn (Asian), Hisp (Hispanic).
†: Due to limited sample volume, only 67 samples were analyzed by the CHL.

<table>
<thead>
<tr>
<th>N</th>
<th>72†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age / y</td>
<td>63 ± 14</td>
</tr>
<tr>
<td>Female:Male</td>
<td>35:37</td>
</tr>
<tr>
<td>BMI / kg/m²</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>Family CVD history</td>
<td>34 (6)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>39 (1)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>58</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>23</td>
</tr>
<tr>
<td>Known CAD</td>
<td>36</td>
</tr>
<tr>
<td>Prior PCI</td>
<td>21</td>
</tr>
<tr>
<td>Prior CABG</td>
<td>8</td>
</tr>
<tr>
<td>Total cholesterol / mg/dL</td>
<td>172 ± 48</td>
</tr>
<tr>
<td>LDL / mg/dL</td>
<td>98 ± 37</td>
</tr>
<tr>
<td>HDL / mg/dL</td>
<td>46 ± 22</td>
</tr>
<tr>
<td>Aspirin</td>
<td>49</td>
</tr>
<tr>
<td>β-blockers</td>
<td>41 (1)</td>
</tr>
<tr>
<td>Statins</td>
<td>39</td>
</tr>
<tr>
<td>P2Y₁₂ inhibitors</td>
<td>21</td>
</tr>
<tr>
<td>Heparin</td>
<td>17</td>
</tr>
<tr>
<td>ELISA MPO / µg/L</td>
<td>20 ± 16</td>
</tr>
<tr>
<td>MAPS MPO / µg/L</td>
<td>14 ± 13</td>
</tr>
</tbody>
</table>

4.3.2 MAPS assay and ELISAs on clinical plasma samples

To determine if the MAPS assay is a suitable alternative to a clinical MPO ELISA kit, plasma samples from the 72 patient cohort were first analyzed in triplicate with both methods. The ELISA kit protocol was modified to be as similar to MAPS as possible, such that the assays were considered “in parallel” in order to isolate disagreement due to the mechanistic differences between MAPS and ELISA. The samples were also analyzed by another ELISA performed at the CHL with no modifications to their proprietary technique. Only 67 of the patient plasma samples had adequate volume for shipment and analysis by the CHL.
Figure 4.2: Calibration curves for ELISA and MAPS assays. WLS calibration curves for (A) ELISA 450 nm absorbance and (B) MAPS bioluminescence used for the initial measurements of 72 clinical plasma samples. (C) Calibration curve from the CHL ELISA used for 67 patient samples with adequate sample volume. Each of the three curves is shown as a dashed line with triplicate 95% prediction intervals shown in gray, darker where independent calibration curves overlap. ELISA calibration curves were consistently and substantially concave while any deviation from linearity in MAPS curves is convex and comparably minor. The concavity resulted in a statistically significant difference in the limits of detection between the two parallel assays: 7.4 µg/L (95% confidence interval 6.7–8.1 µg/L) for ELISA and 6.0 µg/L (95% confidence interval 5.3–6.6 µg/L) for MAPS (p = 0.002). Data from the CHL were provided as molar rather than mass concentration, preventing direct comparison.

The first observable difference between the methods is in calibration curve parameters. Both ELISA curves were moderately concave owing to the saturable nature of absorbance measurements, perhaps additionally due to the concentrated plasma required for signal saturating the available antibody binding sites on the plate surface. This downward concavity was more pronounced near the upper limit of the calibration curve in the CHL data compared to the parallel ELISA. (Figures 4.2A and 4.2C) In contrast, the MAPS assay calibration curves were linear or marginally convex. (Figure 4.2B) This difference favors the MAPS assay, as concavity increases the positive uncertainty of any given assay result. This advantage manifests as a lower limit of detection for MAPS than with the parallel ELISA, 6.0 µg/L (95% confidence interval 5.3–6.6 µg/L) and 7.4 µg/L (95% confidence interval 6.7–8.1 µg/L), respectively (p = 0.002).
Chapter 4: Clinical ELISA and MAPS assays

Results

Figure 4.3: Pearson correlations between MAPS and ELISAs performed on patient samples. Unweighted correlations were computed from the assay estimates for each patient analyzed in triplicate. Sample weights for weighted analysis were calculated by the inverse product of sample uncertainty from the paired assay techniques. This uncertainty was computed by WLS regression. ELISA in this analysis refers to the benchtop kit performed in parallel with MAPS. MAPS-ELISA correlation \( N = 72 \); both correlations with CHL \( N = 67 \) (reduced due to limited volumes).

Although data from the CHL are not in the same unit dimensions as the MAPS and parallel ELISA results, they can be compared with correlation. WLS regression with the two assays resulted in highly correlated MPO estimates (Pearson \( r = 0.969 \)). The CHL assay correlated with the parallel ELISA more than with the MAPS assay (\( r = 0.893 \) and 0.860, respectively) but it was still the outlier. Adjusting for the uncertainty in results from each assay by weighting the each regressed value by the inverse product of the compared method’s uncertainty did not change this trend; MAPS and the parallel ELISA correlated more with each other than either did with the CHL ELISA. (Figure 4.3)

The assay techniques were also compared by testing for systematic differences in their results. Across the 72-patient cohort, MAPS and the parallel ELISA were not significantly different by paired comparison (\( p = 0.46 \)). In order to determine if this lack of disagreement was due to combined uncertainty, each patient sample was again weighted by the inverse product of the confidence interval magnitude and tested again as paired outcomes; this resulted in even less significance than the unweighted test (\( p = 0.61 \)). (Figure 4.4A)

This comparison could not be repeated with MAPS and CHL results as the CHL data were in molar concentration instead of mass concentration, but the data from the CHL
Figure 4.4: Clinical plasma MPO measured by ELISAs and MAPS. (A) Regressed plasma MPO from 72 clinical plasma samples, each measured in triplicate. Error bars represent 95% confidence intervals constructed from combined calibration curve- and inter-replicate-uncertainty as determined by WLS regression. The dashed line shows perfect 1:1 agreement between the two assays. Collectively, assay results were not significantly different from this perfect agreement by either unweighted \( p = 0.46 \) or uncertainty-weighted \( p = 0.61 \) regression and were strongly correlated by unweighted \( r = 0.969 \) and weighted \( r = 0.960 \) analysis. (B) Similarly regressed MPO from 67 patient samples with adequate volume for analysis by the CHL. Paired statistical comparison was limited due to the imprecision in reported MPO molecular weight far exceeding the imprecision in regressed values. The two assays were still well correlated by unweighted \( r = 0.860 \) and weighted \( r = 0.895 \) analysis.

and MAPS assays are nonetheless correlated with unweighted and weighted \( r = 0.860 \) and 0.895, respectively. (Figure 4.4B) The CHL data can be compared to the MAPS and parallel ELISA data together, however, by estimating MPO to be 150,000 kDa for conversion to mass-concentration. Although this approximation is too rough for direct comparison of either parallel assay to the CHL data, the differences between CHL and both methods can be compared directly; the correction term cancels and thus does not have a substantial effect on the result. The disagreement between CHL and MAPS was not significantly different.
Figure 4.5: CHL disagreement with MAPS and parallel ELISA. Results from the CHL assay were roughly converted to mass-concentration before each patient sample discrepancy was calculated for both MAPS and the parallel ELISA. The two parallel assays agreed more with each other than either did with CHL, as demonstrated by the dashed line representing perfect agreement. Generalized linear regression failed to demonstrate significant difference between the disagreements of either parallel assay with the CHL results ($p = 0.20$).

Despite the agreement across all patient samples, not all individual plasma measurements agreed within the 95% confidence limits of the two parallel assays. The mean difference (ELISA – MAPS) with 95% coverage was 2.3 ($-9.2$ to $14$) µg/L MPO. There was no MPO-dependent statistical trend in the discrepancy. (Figure 4.6A) Uncertainty in the MPO measurements, which incorporates variance from both the calibration curve and individual plasma sample measurements, was also consistently distributed between the two assays. The difference ($\Delta$ELISA – $\Delta$MAPS) in assay uncertainty with 95% coverage was $-2.1$ ($-14$ to $10$) µg/L MPO. (Figure 4.6B)
4.3.3 MAPS assay reproducibility

A common and important metric of assay utility is the absolute and relative dispersion of independent results. The MAPS assay was reproduced independently in triplicate for each of the 72 plasma samples over three days and the absolute ($\bar{x}_q$) and relative ($\omega$) variability was quantified according to the method described in Appendix Section §7.3. Median variability was 0.67 µg/L MPO and 95% of assays reproducible within 5.8 µg/L without considering the MPO-dependence of replication dispersion. OLS linear regression of variability with respect to uncertainty-weighted replicate mean resulted in a proportion 0.119 (95% confidence interval 0.106–0.131), predicting a coefficient of variability of ~10%. (Figure 4.7A) Computing the coefficient of variability directly agreed with this prediction with a median $\omega = 8.6\%$ with 95th percentile coverage by 27%. This one-tailed limit was high owing to assay results near the limits of detection, but they were retained in the analysis so as not to subject the metrics
Figure 4.7: MAPS assay reproducibility. (A) Replication variability $\bar{s}_q$ from four independent MAPS assay replicates each performed in triplicate. Median variability was 0.67 µg/L MPO with 95% of assays reproducible within 5.8 µg/L. Deviation was proportional to measured MPO by a ratio of 0.119 (95% confidence interval 0.106–0.131). Dashed lines are the mean linear relationship of assay MPO ± 95% prediction interval. (B) Replication reproducibility $\bar{s}_q$ normalized to variance-weighted mean MPO $\bar{x}_q$, giving a more robust version of the coefficient of variation ($\omega$). Median variation was 8.6% with 95% assays within 27% relative variation, represented by dashed lines. These values were strongly affected by samples near the limits of assay detection, but these remained in the analysis dataset to prevent experimenter bias.

to researcher bias. (Figure 4.7B) These numbers cannot be compared to ELISA as this was prohibited by cost.

4.4 Discussion

MPO is a potentially useful CVD risk stratification biomarker and a prognostic indicator for patients presenting with major adverse cardiovascular events (MACEs). This study shows that human plasma MPO can be assayed by a new method, the MAPS assay, as an alternative to the costly and time-intensive ELISAs used in the clinical setting today. One of the potential advantages of a point-of-care MPO assay is its predictive value over short timespans, especially when utilizing serial measurements. The MAPS assay described herein
Chapter 4: Clinical ELISA and MAPS assays

Discussion

improves greatly upon ELISA in terms of cost and required person-hours in addition to its greatly reduced complexity and plasma sample volume requirement.

The means of measurement in the MAPS assay fundamentally differs from that of ELISA, which relies on immunoconjugated horseradish peroxidase (HRP) to measure the mass concentration of MPO bound to adsorbed immunoglobulins. The MAPS assay instead measures MPO activity through its halogenation-dependent bioluminescence of L-012 (see Section §2.3.3, page 53). Each mechanism has potential disadvantages. The MAPS assay assumes enzyme activity equivalence between plasma MPO and the purified MPO added to RBP for the standard solutions. Hypomorphic MPO due to a genetic polymorphism could result in underestimated MPO concentration. ELISA is subject to a similar problem, but with antigenic variation leading to underestimated assay results. Additionally, ELISA can also be affected by sample MPO as a result of what could be considered a critical assay design flaw: MPO and HRP can both catalyze the reaction responsible for the change in 450 nm absorbance. Thus ELISA ΔAbs$_{450}$ is not a linear function of MPO concentration; it is an integration of MPO activity, HRP activity, and the combined affinity of two different antibodies for sample MPO. This study did not identify any patients with highly discrepant MPO results from the two assays. Given the estimated 0.05–0.15% rate of hypomorphic MPO, large-scale clinical trials with the MAPS assay should include either genetic screening for MPO deficiency or a whole-blood bioluminescence assay (see Section §2.3.1, page 48).$^{43-45}$

ELISA is a well-validated and accepted method of protein quantification in both basic science research and clinical laboratories responsible for guiding patient care. From this, it is reasonable to consider any disagreement between equally validated ELISAs to be within acceptable limits. Although the technique is considered to be reproducible, many sources of error still affect ELISA results beyond the expected random variation. Systematic error
can emerge from extrinsic sources such as operator technique, handling samples, and bias introduced by the measuring instruments. Error may also come from intrinsic sources; these include epitope frequency, differing antibody affinity, and chemical interactions between the analyte and different assay components. The parallel ELISA was designed to minimize the procedural systematic error between it and MAPS in order to isolate disagreement resulting from the fundamental, mechanistic differences between the two assays, namely measuring adsorbed MPO activity versus immunologically bound MPO concentration. Surprisingly, the parallel assays agreed far more than the two ELISAs did, even though both ELISAs are FDA-approved and suitable as gold standards of MPO concentration. This strongly supports the further development of MAPS as an alternative to MPO ELISA in the clinical setting.

Although similar to ELISA in terms of sensitivity and reproducibility, the MAPS assay has many practical advantages that make it a desirable alternative. Most obvious is the difference in cost: ELISA has numerous biological components that drive raw material costs to > $20 per patient sample run in triplicate. Conversely, the most costly consumable in the MAPS assay is the plastic used to isolate MPO; reagents consumed in a 24-patient assay run in triplicate add no significant cost in comparison; a patient sample can be run in triplicate against 24 standard concentration samples for ~30¢. The lack of biologic reagents is another advantage belonging to the MAPS assay—ELISA kits require 4 °C storage and thus have a very limited shelf-life, while MPO and RBP can be stored for years at −80 °C, and no loss in L-012 stock activity was observed over the >3 years of storage at −20 °C some aliquots endured during the assay development. Additionally, ELISA requires numerous manipulations spanning many hours; the MAPS assay required only one incubation for which 30 min was chosen arbitrarily for consistency despite evidence that 15 min was more than sufficient (see Figure 3.6 on page 100). MAPS also demonstrated increased linearity and sensitivity than
ELISA, in part because luminescence is less saturable than absorbance. (Figure 4.2) Higher sensitivity also allows MAPS to be measured from plasma diluted to 0.4% \( v/v \) compared to 4% \( v/v \) required by standard ELISA, further extending the dynamic range in addition to requiring far less sample material.

Like any assay method, MAPS is not without its disadvantages, both intrinsic and circumstantial. As of the time of this writing, the plasma proteins required for MPO adsorption onto polystyrene are unknown (see Figure 3.11 on page 105). The effect of intravenous heparin, which is common among the patient population with the most to gain from this assay, is also not known. The pilot clinical study did not detect a statistical difference in the \( \text{ELISA} - \text{MAPS} \) discrepancy when comparing the heparin-treated patients to the untreated group, but his trial was neither powered nor designed to detect such a difference. This pilot study demonstrates the fundamental feasibility of the MAPS assay in a clinical setting, but further analysis of outcome predictive value for CVD in the same manner as MPO ELISA is required.

Although the advanced ONYX/M imaging system was used throughout the development of this assay, MAPS can be measured with common laboratory equipment. A feasibility test using a low-sensitivity camera intended to image gel electrophoresis bands was able to resolve the standard curve on a plate set up in parallel with one of the clinical sample blocks. Two microtiter plate luminometers in addition to a single-channel, test tube luminometer also had adequate sensitivity to discriminate MPO luminescence at all levels of the MAPS standard curve. (Data not shown) This establishes the feasibility of further studies in a wide range of clinical settings. The microtiter plate platform served as a convenient and reliable source of hydrophilic polystyrene, but the MAPS assay is amenable to other platforms as well. For example, treated polystyrene in the form of a dipstick could increase the portability
of the MAPS assay to that of blood glucose meters. The work described here focuses on the application to CVD diagnosis and prognosis, but there are other clinical scenarios (e.g., transplant rejection or sepsis) for which a rapid MPO measurement via the MAPS assay could reduce the time between onset and treatment of potentially life-threatening complications.\textsuperscript{46,47}

In conclusion, a facile MPO activity assay was developed for application with clinical plasma samples as an alternative to ELISA. The MAPS assay has many practical advantages including a $>10$-fold reduction in cost, sample material consumption, and labor requirement. Importantly, the MAPS assay was comparable to ELISA in results, uncertainty, and reproducibility in a test with 72 cardiac catheterization patient samples.

4.5 References


Conclusions and Future Directions

5.1 New strategies in CVD management

Atherosclerosis, the disease process behind most cardiovascular disease (CVD), has a larger burden of morbidity, mortality, and health-related economic costs than any other single pathology. With the exception of the 1918 influenza pandemic, this has held true in the United States for the entirety of the last century.\(^1\) As sanitation and infectious disease prevention improve in developing nations, CVD predictably rises in public health statistics to take the same position.\(^2,3\) Through the long history of CVD research, the disease and the atherosclerosis driving it went from being considered a normal part of human physiology, then to a disorder of lipid intake and metabolism, and finally to a systemic inflammatory condition resulting from complex interactions between coagulation, metabolism, hemodynamics, and the immune system.\(^4-9\) Although large atheromas in the coronary arteries can impede blood flow and gradually lead to hypoxia on exertion, the true danger of CVD is the collection of sequelae known as MACEs. MACEs are usually considered to comprise stroke, MI, and ACS; these are results of degradation at the fibrous cap normally covering atheromatous plaques. Without the anti-thrombotic surface maintained by endothelial cells, the coagulation cascade is activated at the inflamed arterial wall. As the thrombus grows, it can completely occlude...
the artery or release thromboemboli that are trapped in the arterial tree as luminal diameter decreases with each subsequent branching. As an unfortunate legacy of our evolutionary heritage, both the human heart and brain are poorly supported by redundant vasculature and anastomoses despite the importance and metabolic demand of each organ. CVD can progress without symptoms for decades until if manifests as a MACE; this has led to increased efforts to develop methods of identifying vulnerable plaques and patients statistically inclined to develop disseminated vascular inflammation so that primary and secondary intervention can slow or halt CVD progression rather than tertiary intervention dominating CVD treatment costs as is true today.\textsuperscript{10,11} Biomarkers for many facets of CVD are being developed, including myocardial strain, hypercoagulability, lipid dysregulation, and inflammation.\textsuperscript{12–15}

\section{Elevated plasma MPO: cause or consequence?}

In the preceding chapters, MPO has been discussed as both a novel biomarker for CVD progression and a possible contributor to the molecular processes behind atheromatous plaque initiation, development, and rupture. Although most published studies show that MPO measured by ELISA is a useful biomarker even after adjusting for established measures of CVD risk, studies failing to demonstrate such utility are also in the literature, albeit in smaller numbers.\textsuperscript{16–30} This contradiction appears to have origins beyond typical publication biases. As pointed out by others, neutrophils are the primary source of plasma MPO and are exquisitely sensitive to activating stimuli. Study methodologies rarely take this into consideration; there is no standard protocol designed to control for the effects of anticoagulants, sample handling, or external reference standardization.\textsuperscript{31–35} At the core of this issue is the scarcity of well-powered, well-designed studies of MPO biomarker utility. This is somewhat understandable, given the high cost of measuring MPO with current, ELISA-based techniques. The work
described in these chapters was intended to be a step toward a solution to this problem. The MAPS assay allows for independent studies on the predictive and prognostic value of MPO activity to be performed by investigators without large, high-profile funding sources. If the results of such studies are favorable, the same economic advantages of the MAPS assay over ELISA in the research setting will apply equally well to the point-of-care, as the population most afflicted by CVD is also the population least able to afford expensive diagnostic tests.

Although not the focus of the work described herein, the causative role MPO may play in CVD pathophysiology can also be investigated by adapting these methods. Animal studies attempting to deduce this role have thus far been conflicting.\textsuperscript{36,37} Human studies into the role of MPO-dependent oxidation in atherosclerosis are even fewer than the biomarkers studies. The MAPS assay and whole-blood L-012 bioluminescence offer powerful new tools in investigating the potential oxidative MPO burden in human and animal models. Examples of specific use include rapidly identifying \textit{MPO}\textsuperscript{+/−} and \textit{MPO}\textsuperscript{−/−} individuals for prospective clinical studies, determining the neutrophil priming effects of stimuli such as smoking or poorly regulated diabetes, and directly imaging atherosclerosis in experimental animal models with L-012.

\section{Further MAPS assay development}

As discussed in Chapter 3, there are remaining questions involving the MAPS assay and the molecular interactions that allow it to be performed. One of the foremost is the interaction or combination of interactions required for MPO adsorption to tissue culture-treated polystyrene. The range of pH that allows this interaction is narrow, indicating a degree of specificity behind it. (see Figure 3.13 on page 109) Although few proteins were tested individually and with no
success, (see Figure 3.11 on page 105) there might be a single protein or specific mixture that allows adsorption with equal affinity to that seen from RBP or human plasma, but with fewer unknown parameters to potentially confound the assay result. The mechanism of the robust bioluminescence enhancing effect seen with concentrated \((\text{NH}_4)_2\text{SO}_4\) added to adsorbed MPO is also unknown and presents another area of future research. (see Figure 3.15 on page 114) This could not only have implications in further development of the MAPS assay, but may also provide new insight into the biochemistry of MPO and related oxidoreductase enzymes.

Although this work, specifically that in Chapter 4, describes a pilot clinical study comparing the MAPS assay to ELISA measurements, there has yet to be an investigation into the independent predictive value of plasma MPO bioluminescence for CVD outcomes. Even if MAPS proves only as predictive as literature reports of plasma MPO concentration measured by ELISA, the aforementioned economic advantages would be sufficient to give MAPS utility beyond that of immunologic techniques. Additionally, there is potential for MPO activity to be an improved prognostic indicator compared to MPO concentration, especially considering the possible causative role the ROS produced by MPO may play in atherogenesis. Investigations into new platforms, such as portable dipsticks or disposable biosensors, further open the concept behind the MAPS assay to a diverse range of research possibilities. To further encourage development of the MAPS assay and investigation into its utility in other clinical applications, the work described here was published in an open access journal.\(^{38}\)
5.4 References


Appendix
## List of chemical and reagent aliases

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
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<td>4-ABH</td>
<td>4-aminobenzoic hydrazide</td>
</tr>
<tr>
<td>AcDPBS</td>
<td>pH = 6.0 DPBS with 5 mM acetic acid, pH adjusted with NaOH</td>
</tr>
<tr>
<td>B(OH)$_3$</td>
<td>Boric acid</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-(cyclohexylamino)-1-propanesulfonic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>BLI-6</td>
<td>MAPS imaging solution, see Table 4.1</td>
</tr>
<tr>
<td>ioflurane</td>
<td>2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane</td>
</tr>
<tr>
<td>L-012</td>
<td>8-amino-5-chloro-7-phenyl-2,3-dihydropyrido[3,4-d]pyridazine-1,4-dione</td>
</tr>
<tr>
<td>luminol</td>
<td>5-amino-2,3-dihydrophthalazine-1,4-dione</td>
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<tr>
<td>MEBSS</td>
<td>Modified Earle's balanced salt solution</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
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<tr>
<td>PS-6.5</td>
<td>MAPS loading/wash buffer, see Table 4.1</td>
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<td>PS-6.5$^T$</td>
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<tr>
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<td>Polyvinylidene fluoride</td>
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<tr>
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<td>Superoxide</td>
</tr>
<tr>
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<td>Tween20</td>
<td>Polysorbate 20, polyoxyethylene (20) sorbitan monolaurate</td>
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List of source code

7.1 WLS regression .................................................. 212
7.2 Calling WLS regression ........................................... 223
7.3 Quadrangular ROI array .......................................... 226
7.4 ONYX imaging vignette correction ......................... 231
6.1 Contribution to authorship

I participated in a multi-group collaboration to analyze full genomic, expression, and clinical outcome data from a cohort of patients undergoing aromatase inhibition therapy as a treatment for breast cancer. My specific contribution involved the annotation and analysis of gene networks disproportionately affected by \textit{de novo} mutations found in solid tumor biopsies compared against a matched, normal skin genome. Over the course of the project, the primary contributing authors met many times every month to discuss progress, complications, and new developments in the ongoing data analyses, patient recruitment, and conclusions of the project. I contributed to the manuscript by writing the main-text section on gene network analysis, the corresponding methods and discussion found in the online supplement, compiling and statistically analyzing a myriad annotated network maps overrepresented in the mutation dataset, and producing two figures. (Figure 6.4B and Supplementary Figure 6.6)
The full text of the manuscript is reproduced below, edited only for consistency in figure numbering, table formatting, and bibliographic information.\(^1\) References to supplemental information omitted from this chapter due to space are preserved from the original publication.\(^2\)

### 6.2 Published manuscript


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\(^2\)Supplemental material can be found at [http://www.nature.com/nature/journal/v486/n7403/extref/nature11143-s1.pdf](http://www.nature.com/nature/journal/v486/n7403/extref/nature11143-s1.pdf)
6.2.1 Abstract

To correlate the variable clinical features of oestrogen-receptor-positive breast cancer with somatic alterations, we studied pretreatment tumour biopsies accrued from patients in two studies of neoadjuvant aromatase inhibitor therapy by massively parallel sequencing and analysis. Eighteen significantly mutated genes (SMGs) were identified, including five genes \( \text{RUNX1, CBFB, MYH9, MLL3 and SF3B1} \) previously linked to haematopoietic disorders. Mutant \( \text{MAP3K1} \) was associated with luminal A status, low-grade histology and low proliferation rates, whereas mutant \( \text{TP53} \) was associated with the opposite pattern. Moreover, mutant \( \text{GATA3} \) correlated with suppression of proliferation upon aromatase inhibitor treatment. Pathway analysis demonstrated that mutations in \( \text{MAP2K4} \), a \( \text{MAP3K1} \) substrate, produced similar perturbations as \( \text{MAP3K1} \) loss. Distinct phenotypes in oestrogen-receptor-positive breast cancer are associated with specific patterns of somatic mutations that map into cellular pathways linked to tumour biology, but most recurrent mutations are relatively infrequent. Prospective clinical trials based on these findings will require comprehensive genome sequencing.

6.2.2 Introduction

Oestrogen-receptor-positive breast cancer exhibits highly variable prognosis, histological growth patterns and treatment outcomes. Neoadjuvant aromatase inhibitor treatment trials provide an opportunity to document oestrogen-receptor-positive breast cancer phenotypes in a setting where sample acquisition is easy, prospective consent for genomic analysis can be obtained and responsiveness to oestrogen deprivation therapy is documented.\(^1\) We therefore conducted massively parallel sequencing (MPS) on 77 samples accrued from two neoadjuvant aromatase inhibitor clinical trials.\(^2,3\) Forty-six cases underwent whole-genome
sequencing (WGS) and 31 cases underwent exome sequencing, followed by extensive analysis for somatic alterations and their association with aromatase inhibitor response. Case selection for discovery was based on the levels of the tumour proliferation marker Ki67 in the surgical specimen, because high cellular proliferation despite aromatase inhibitor treatment identifies poor prognosis tumours exhibiting oestrogen-independent growth\textsuperscript{4} (Supplementary Figure 1). Twenty-nine samples had Ki67 levels above 10% (‘aromatase-inhibitor-resistant tumours’, median Ki67 21%, range 10.3–80%) and 48 were at or below 10% (‘aromatase-inhibitor-sensitive tumours’, median Ki67 1.2%, range 0–8%). Cases were also classified as luminal A or B by gene expression profiling.\textsuperscript{3} We subsequently examined interactions between Ki67 biomarker change, histological categories, intrinsic subtype and mutation status in selected recurrently mutated genes in 310 cases overall. Pathway analysis was applied to contrast the signalling perturbations in aromatase-inhibitor-sensitive versus aromatase-inhibitor-resistant tumours.

6.2.3 Results

The mutation landscape of luminal-type breast cancer

Using paired-end MPS, 46 tumour and normal genomes were sequenced to at least 30-fold and 25-fold haploid coverage, respectively, with diploid coverage of at least 95% based on concordance with SNP array data (Supplementary Table 1). Candidate somatic events were identified using multiple algorithms,\textsuperscript{5,6} and were then verified by hybridization capture-based validation that targeted all putative somatic single nucleotide variants (SNVs) and small insertions/deletions (indels) that overlap coding exons, splice sites and RNA genes (tier 1), high-confidence SNVs and indels in non-coding conserved or regulatory regions (tier 2), as well as non-repetitive regions of the human genome (tier 3).
addition, somatic structural variants and germline structural variants that potentially affect coding sequences (Supplementary Information) were assessed. Digital sequencing data from captured target DNAs from the 46 tumour and normal pairs (Supplementary Table 2 and Supplementary Information) confirmed 81,858 mutations (point mutations and indels) and 773 somatic structural variants. The average numbers of somatic mutations and structural variants were 1,780 (range 44–11,619) and 16.8 (range 0–178) per case, respectively (Supplementary Table 3). Tier 1 point mutations and small indels predicted for all 46 cases also were validated using both 454 and Illumina sequencing (Supplementary Information). BRC25 was a clear outlier with only 44 validated tiers 1-3 mutations, all at low allele frequencies (ranging from 5% to 26.8%). This sample probably had low tumour content despite histopathology assessment, but the data are included to avoid bias.

The overall mutation rate was 1.18 validated mutations per megabase (Mb) (tier 1: 1.05; tier 2: 1.14; tier 3: 1.20). The mutation rate for tier 1 was higher than that observed for acute myeloid leukaemia (0.18–0.23),6,7 but lower than that reported for hepatocellular carcinoma (1.85),8 malignant melanoma (6.65)9 and lung cancers (3.05–8.93).10,11 (Supplementary Table 4) The background mutation rate across the 21 aromatase-inhibitor-resistant tumours was 1.62 per Mb, nearly twice that of the 25 aromatase-inhibitor-sensitive tumours at 0.824 per Mb (P = 0.02, one-sided t-test). A trend for more somatic structural variations in the aromatase-inhibitor-resistant group was also observed, as the validated somatic structural variation frequency in the 21 aromatase-inhibitor-resistant tumour genomes was 21.69 versus an average of 12.76 in 25 aromatase-inhibitor-sensitive tumours (P = 0.16, one-sided t-test). (Figure 6.1) If ten TP53 mutated cases were excluded, the background mutation rate still tended to be higher in the aromatase-inhibitor-resistant group (P = 0.08). To demonstrate that a single-tumour core biopsy produced representative
genomic data, whole-genome sequencing of two pre-treatment biopsies was conducted for 5 of the 46 cases. The frequency of mutations in the paired specimens showed high concordance in all cases (correlation coefficient ranged from 0.74 to 0.95) and a somatic mutation was infrequently detected in only one of the two samples (4.65% overall). (Supplementary Figure 2)
Significantly mutated genes in luminal breast cancer

The discovery effort was extended by studying 31 additional cases by exome sequencing, producing an additional 1,371 tier 1 mutations. In total the 77 cases yielded 3,355 tier 1 somatic mutations, including 3,208 point mutations, 1 dinucleotide mutation and 146 indels, ranging from 1 to 28 nucleotides. Of 2,145 missense mutations, 1,551 were predicted to be deleterious by SIFT and/or PolyPhen.\textsuperscript{13,14} The MuSiC package was applied to determine the significance of the difference between observed versus expected mutation events in each gene, on the basis of the background mutation rate.\textsuperscript{15} This identified 18 SMGs with a convolution false discovery rate (FDR) < 0.26. (Table 6.1 and Supplementary Table 6) The list contains genes previously identified as mutated in breast cancer (\textit{PIK3CA}, \textit{TP53}, \textit{GATA3}, \textit{CDH1}, \textit{RB1}, \textit{MLL3}, \textit{MAP3K1} and \textit{CDKN1B}) as well as genes not previously observed in clinical breast cancer samples, including \textit{TBX3}, \textit{RUNX1}, \textit{LDLRAP1}, \textit{STNM2}, \textit{MYH9}, \textit{AGTR2}, \textit{STNM2}, \textit{SF3B1}, and \textit{CBFB}.\textsuperscript{13,14,16–21}

Thirteen mutations (3 nonsense, 6 frame-shift indels, 2 in-frame deletions and 2 missense) were identified in \textit{MAP3K1}, a serine/threonine kinase that activates the ERK and JNK kinase pathways through phosphorylation of \textit{MAP2K1} and \textit{MAP2K4}.\textsuperscript{22} (Table 6.1 and Figure 6.2) Of interest, a missense (S184L) and a splice-region mutation (e2+3 probably affecting splicing) in \textit{MAP2K4} were observed in two tumours with no \textit{MAP3K1} mutation. (Figure 6.2) Single nonsynonymous mutations in \textit{MAP3K12}, \textit{MAP3K4}, \textit{MAP4K3}, \textit{MAP4K4}, \textit{MAPK15}, and \textit{MAPK3} were also detected. (Supplementary Table 5) \textit{TBX3} harboured three small indels (one insertion and two deletions). \textit{TBX3} affects expansion of breast cancer stem-like cells through regulation of FGFR.\textsuperscript{23} Two truncating mutations in the tumour suppressor \textit{CDKN1B} were identified.\textsuperscript{21} Four missense \textit{RUNX1} mutations were observed, with three in the RUNT domain clustered within the 8 amino acid putative ATP-binding site
(R166Q, G168E and R169K). RUNX1 is a transcription factor affected by mutation and translocation in the M2 subtype of acute myeloid leukaemia and is implicated in tethering the oestrogen receptor to promoters independently of oestrogen response elements.\textsuperscript{24,25} Two mutations (N104S and N140*) were also identified in CBFB, the binding partner of RUNX1. Additional mutations included 3 missense (2 K700E and 1 K666Q), in SF3B1, a splicing factor implicated in myelodysplasia and chronic lymphocytic leukaemia.\textsuperscript{26,27} One missense mutation, one nonsense mutation and two indels were found in the MYH9 gene, involved in hereditary macrothrombocytopenia as well as being observed in an ALK translocation in anaplastic large cell lymphoma.\textsuperscript{28,29}

We also identified three SMGs (LDLRAP1, AGTR2, and STMN2) not previously implicated in cancer. A missense and a nonsense mutation were observed in LDLRAP1, a gene associated with familial hypercholesterolaemia.\textsuperscript{30} AGTR2, angiotensin II receptor type 2, harboured two missense mutations (V184I and R251H). Angiotensin signalling and oestrogen receptor intersect in models of tissue fibrosis.\textsuperscript{31} STMN2, a gene activated by JNK family kinases and therefore regulated by MAP3K1 and MAP2K4, harboured one frameshift deletion and one missense mutation.\textsuperscript{32,33} Three deletions and one point mutation were identified in a large, infrequently spliced non-coding (Inc) RNA gene, MALAT1 (metastasis associated lung adenocarcinoma transcript 1), that regulates alternative splicing by modulating the phosphorylation of SR splicing factor.\textsuperscript{34} (Supplementary Figure 3) Translocations and point mutations of MALAT1 have been reported in sarcoma and colorectal cancer cell lines.\textsuperscript{35,36} Five additional MALAT1 mutations were found in the recurrent screening set. (Supplementary Table 5d) The locations of these mutations clustered in a region of species homology (F1 and 2 domains) that could mediate interactions with SRSF1.\textsuperscript{34}
Table 6.1: Significantly mutated genes identified in 46 whole genomes and 31 exomes sequenced in luminal breast cancer patients.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total</th>
<th>MS</th>
<th>NS</th>
<th>Indel</th>
<th>SS</th>
<th>P value</th>
<th>FDR</th>
</tr>
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<tbody>
<tr>
<td>MAP3K1</td>
<td>13</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>45</td>
<td>44</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TP53</td>
<td>18</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GATA3</td>
<td>8</td>
<td>1</td>
<td>–</td>
<td>4</td>
<td>3</td>
<td>1.15 x 10^{-19}</td>
<td>7.41 x 10^{-16}</td>
</tr>
<tr>
<td>CDH1</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>3.07 x 10^{-15}</td>
<td>1.59 x 10^{-11}</td>
</tr>
<tr>
<td>TBX3</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>–</td>
<td>2.58 x 10^{-6}</td>
<td>0.011</td>
</tr>
<tr>
<td>ATR</td>
<td>6</td>
<td>6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.73 x 10^{-6}</td>
<td>0.014</td>
</tr>
<tr>
<td>RUNX1</td>
<td>4</td>
<td>4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>6.59 x 10^{-6}</td>
<td>0.021</td>
</tr>
<tr>
<td>ENSG00000212670*</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.31 x 10^{-5}</td>
<td>0.066</td>
</tr>
<tr>
<td>RB1</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>2.76 x 10^{-5}</td>
<td>0.071</td>
</tr>
<tr>
<td>LDLRAP1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>4.27 x 10^{-5}</td>
<td>0.092</td>
</tr>
<tr>
<td>STMN2</td>
<td>2</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>4.15 x 10^{-5}</td>
<td>0.092</td>
</tr>
<tr>
<td>MYH9</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>–</td>
<td>8.96 x 10^{-5}</td>
<td>0.178</td>
</tr>
<tr>
<td>MLL3</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>–</td>
<td>1.04 x 10^{-4}</td>
<td>0.191</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>2</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>1.39 x 10^{-4}</td>
<td>0.24</td>
</tr>
<tr>
<td>AGTR2</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.71 x 10^{-4}</td>
<td>0.256</td>
</tr>
<tr>
<td>SF3B1</td>
<td>3</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.79 x 10^{-4}</td>
<td>0.256</td>
</tr>
<tr>
<td>CBFB</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>1.70 x 10^{-4}</td>
<td>0.256</td>
</tr>
</tbody>
</table>

* ENSG00000212670 is not in RefSeq release 50.

MS, Missense; NS, nonsense; SS, splice site.

(Supplementary Figure 4) Non-coding mutation clusters were found in ATR, GPR126 and NRG3. (Supplementary Information and Supplementary Table 7)

Correlating mutations with clinical data

To study clinical correlations, mutation recurrence screening was conducted on an additional 240 cases. (Supplementary Table 8 and Supplementary Figure 1) By combining WGS, exome and recurrence screening data, we determined the mutation frequency in PIK3CA to be 41.3% (131 of 317 tumours). (Supplementary Table 5a–d and Supplementary Figure 3) TP53 was mutated in 51 of 317 tumours (16.1%). (Supplementary Table 5a–d and Supplementary Figure 3) Additionally, 52 nonsynonymous MAP3K1 mutations in 39 tumours and 10 mutations in its substrate MAP2K4 were observed, representing a combined case
Figure 6.2: MAP3K1 and MAP2K4 mutations observed in 317 samples. Somatic status of all mutations was obtained by Sanger sequencing of PCR products or Illumina sequencing of targeted capture products. The locations of conserved protein domains are highlighted. Each nonsynonymous substitution, splice site mutation or indel is designated with a circle at the representative protein position with colour to indicate translation effects of the mutation. Asterisk, nonsense mutations that cause truncation of the open reading frame.
frequency of 15.5%. (Supplementary Table 5a-d and Figure 6.3) Of note, 52 of the 62 non-silent mutations in \textit{MAP3K1} and \textit{MAP2K4} were scattered indels or other protein-truncating events strongly suggesting functional inactivation. In addition, 13 tumours harboured two non-silent \textit{MAP3K1} mutations, indicative of bi-allelic loss and reinforcing the conclusion that this gene is a tumour suppressor. Twenty nine tumours harboured a total of 30 mutations in \textit{GATA3}, consisting of 25 truncation events, one in-frame insertion, and 4 missense mutations including 3 recurrent mutations at M294K. (Supplementary Table 5a-d and Supplementary Figure 3) BRC8 harboured a chromosome 10 deletion that includes \textit{GATA3}. \textit{CDH1} mutation data were available for 169 samples and, as expected, its mutation status was strongly associated with lobular breast cancer.\(^1\) (Table 6.2A) We applied a permutation-based approach in MuSiC to ascertain relationships between mutated genes.\(^5\) Negative correlations were found between mutations in gene pairs such as \textit{GATA3} and \textit{PIK3CA} (\(P = 0.0026\)), \textit{CDH1} and \textit{GATA3} (\(P = 0.015\)), and \textit{CDH1} and \textit{TP53} (\(P = 0.022\)). \textit{MAP3K1} and \textit{MAP2K4} mutations were mutually exclusive, albeit without reaching statistical significance (\(P = 0.3\)). In contrast, a positive correlation between \textit{MAP3K1}/\textit{MAP2K4} and \textit{PIK3CA} mutations was highly significant (\(P = 0.0002\)). (Supplementary Table 9)

Two independent mutation data sets, designated ‘Set 1’ (discovery cohort) and ‘Set 2’ (validation cohort), from these clinical trial samples were analysed separately and then in combination, with a FDR-corrected \(P\) value to gauge the overall strength and consistency of genotype-phenotype relationships. (Table 6.2A, Table 6.2B, and Supplementary Figure 1) \textit{TP53} mutations in both data sets correlated with significantly higher Ki67 levels, both at baseline (\(P = 0.0003\)) and at surgery (\(P = 0.001\)). Furthermore, \textit{TP53} mutations were significantly enriched in luminal B tumours (\(P = 0.04\)) and in higher histological grade tumours (\(P = 0.02\)). In contrast, \textit{MAP3K1} mutations were more frequent in luminal A
Figure 6.3: Structural variants in significantly mutated or frequently deleted genes. One MAP3K1 deletion in BRC49 and one MAP2K4 deletion in BRC47, and one ELP3-NRG1 fusion in BRC49 identified using Illumina paired-end reads from whole-genome sequence data. Arcs represent multiple breakpoint-spanning read pairs with sequence coverage depth plotted in black across the region. Chr, chromosome.
Table 6.2A: Correlations between mutations and clinical features—Luminal subtype and histology grade.

*Mutation percentage (mutant cases/total cases in a category), counts are based on all cases (Set 1 and Set 2 combined).
†Unadjusted P value from Fisher’s exact test or Chi-square test as appropriate.
‡Benjamini-Hochberg FDR-adjusted P value using all cases (Set 1 and Set 2 combined).
§Only 77 cases in Set 1 had CDH1 sequencing results.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression/histopathology</th>
<th>Mutation frequency*</th>
<th>Set 1 P†</th>
<th>Set 2 P‡</th>
<th>Whole set FDR P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>Luminal subtype A</td>
<td>9.3% (13/140)</td>
<td>0.001</td>
<td>0.46</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td>Luminal subtype B</td>
<td>21.5% (38/177)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td>Histological grade I</td>
<td>4.5% (3/66)</td>
<td>0.05</td>
<td>0.067</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Histological grade II/III</td>
<td>19.2% (48/250)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP3K1</td>
<td>Luminal subtype A</td>
<td>20.0% (28/140)</td>
<td>0.018</td>
<td>0.028</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Luminal subtype B</td>
<td>6.2% (11/177)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP3K1</td>
<td>Histological grade I</td>
<td>25.8% (17/66)</td>
<td>0.061</td>
<td>0.011</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Histological grade II/III</td>
<td>8.8% (22/250)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDH1</td>
<td>Histological type ductal</td>
<td>5.9% (10/169)</td>
<td>0.411§</td>
<td>2.8 × 10⁻¹¹</td>
<td>3.9 × 10⁻¹⁰</td>
</tr>
<tr>
<td></td>
<td>Histological type lobular</td>
<td>50.0% (20/40)</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 6.2B: Correlations between mutations and clinical features—Mutation and Ki67 index.

*Geometric means are based on all cases (Set 1 and Set 2 combined).
†Unadjusted P value from Wilcoxon rank sum test.
‡Benjamini-Hochberg FDR-adjusted P value using all cases (Set 1 and Set 2 combined).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ki67 variable</th>
<th>Wild type mean*</th>
<th>Mutant mean*</th>
<th>Set 1 P†</th>
<th>Set 2 P‡</th>
<th>Whole set FDR P‡</th>
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</thead>
<tbody>
<tr>
<td>TP53</td>
<td>Baseline</td>
<td>13.1</td>
<td>25.1</td>
<td>3.7 × 10⁻⁵</td>
<td>0.012</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Surgery</td>
<td>1.4</td>
<td>4</td>
<td>0.0002</td>
<td>0.014</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>% change</td>
<td>−89.2</td>
<td>−84.3</td>
<td>0.09</td>
<td>0.28</td>
<td>0.24</td>
</tr>
<tr>
<td>MAP3K1</td>
<td>Baseline</td>
<td>15.8</td>
<td>8.1</td>
<td>0.049</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Surgery</td>
<td>1.86</td>
<td>0.75</td>
<td>0.11</td>
<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>% change</td>
<td>−88.3</td>
<td>−90.5</td>
<td>0.49</td>
<td>0.65</td>
<td>0.55</td>
</tr>
<tr>
<td>GATA3</td>
<td>Baseline</td>
<td>14.8</td>
<td>11.5</td>
<td>0.13</td>
<td>0.95</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Surgery</td>
<td>1.95</td>
<td>0.38</td>
<td>0.001</td>
<td>0.23</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>% change</td>
<td>−86.8</td>
<td>−96.9</td>
<td>0.003</td>
<td>0.08</td>
<td>0.012</td>
</tr>
</tbody>
</table>
tumours ($P = 0.02$), in grade 1 tumours ($P = 0.005$) and in tumours with lower Ki67 at baseline ($P = 0.001$) with consistent findings across both data sets. \textit{GATA3} mutation did not influence baseline Ki67 levels but was enriched in samples exhibiting greater percentage Ki67 decline ($P = 0.01$). This finding requires further verification because it was significant in Set 1 (uncorrected $P$ value 0.003) but was a marginal finding in Set 2 ($P = 0.08$). However, it suggests \textit{GATA3} mutation may be a positive predictive marker for aromatase inhibitor response.

\section*{Structural variation and DNA repair mechanisms}

Analysis of copy number alterations (CNAs) revealed arm-level gains for 1q, 5p, 8q, 16p, 17q, 20p and 20q and arm-level losses for 1p, 8p, 16q, and 17p in the 46 WGS tumour genomes. (Supplementary Figure 5) A total of 773 structural variants (579 deletions, 189 translocations and 5 inversions) identified by WGS were validated as somatic in 46 breast cancer genomes by capture validation. No recurrent translocations were detected but six in-frame fusion genes were validated by reverse transcription followed by PCR (Supplementary Information and Supplementary Tables 10–13). Seven tumours had multiple complex translocations with breakpoints suggestive of a catastrophic mitotic event (‘chromothripsis’). (Supplementary Table 11) Analysis of the structural variant genomic breakpoints shows the spectra of putative chromothripsis-related events are the same as seen for other somatic events, with the majority of structural variants arising from non-homologous end-joining. We classified somatic (mitotic) and germline (meiotic) structural variants into four groups: variable number tandem repeat, non-allelic homologous recombination, microhomology-mediated end joining, and non-homologous end joining (NHEJ), according to criteria described in Supplementary Information. The fraction of each classification is shown for germline and somatic (mitotic)
events. (Supplementary Table 14) There were significantly more somatic NHEJ events in tumour genomes than the other three types ($P < 2.2 \times 10^{-16}$).

**Pathways relevant to aromatase inhibitor response**

Pathscan analysis (Supplementary Table 15 and Supplementary Information) indicated that somatic mutations detected in the 77 discovery cases affect a number of pathways, including caspase cascade/apoptosis, ErbB signalling, Akt/PI3K/mTOR signalling, TP53/RB signalling and MAPK/JNK pathways.\(^{37}\) (Figure 6.4A) To discern the pathways relevant to aromatase inhibitor sensitivity, we conducted separate pathway analyses for aromatase-inhibitor-sensitive versus aromatase-inhibitor-resistant tumours. Whereas the majority of top altered pathways (FDR $\leq 0.15$) in each group are shared, several pathways were enriched in the aromatase-inhibitor-resistant group, including the TP53 signalling pathway, DNA replication, and mismatch repair. Specifically, 38% of the aromatase-inhibitor-resistant group (11 of 29 tumours) have mutations in the TP53 pathway with three having double or triple hits involving \(TP53\), \(ATR\), \(APAF1\), or \(THBS1\). In contrast, only 16.6% (8 of 48 tumours) of the Ki67 low group had mutations in the TP53 signalling pathway, each with only a single hit in genes \(TP53\), \(ATR\), \(CCNE2\), or \(IGF1\). (Supplementary Table 16)

GeneGo pathway analysis of MetaCore interacting network objects was used to identify genes in the 77 luminal breast cancers with low-frequency mutations that cluster into pathway maps. Eight networks assembled from significant maps encompassed mutations from 71 (92%) of the tumours. (Figure 6.4B) Many of the network objects shared pathways with SMGs such as \(TP53\), \(MAP3K1\), \(PIK3CA\), and \(CDH1\). GeneGo analysis also revealed that several genes with low-frequency mutations were actually subunits of complexes, resulting in higher mutation rates for that object, for example, the condensin complex (4 mutations in 4
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genes) and the MRN complex (4 mutations in 3 genes). Several pathways without multiple SMGs, such as the apoptotic cascade, calcium/phospholipase signalling and G-protein-coupled receptors, were significantly affected by low-frequency mutations. Grouping tumours by SMGs and pathway mutation status showed that whereas 55 (71%) of the tumours contained SMGs in significant pathways, an additional 16 (21%) contained only non-SMGs in these pathways. Thus, tumours without a given SMG often had other mutations in the same relevant pathway. (Figure 6.4B, Supplementary Figure 6, Supplementary Table 17 and Supplementary Information)

We also applied PARADIGM to infer pathway-informed gene activities using gene expression and copy-number data to identify several ‘hubs’ of activity.\(^{38}\) (Supplementary Figure 7, Supplementary Figure 8 and Supplementary Information) As expected, \(ESR1\) and \(FOXA1\) were among the hubs activated cohort-wide while other hubs exhibited high but differential changes in aromatase-inhibitor-resistant tumours including \(MYC\), \(FOXM1\), and \(MYB\). (Supplementary Figure 8) The concordance among the 104 MetaCore maps from GeneGo analysis described above is significant, with 75 (72%) matching one of the PARADIGM subnetworks at the 0.05 significance level after multiple test correction \((P < 4.4 \times 10^{-6}; \text{Bonferroni-adjusted hypergeometric test})\). (Supplementary Figure 9) We identified significant subnetworks associated with Ki67 biomarker status involving transcription factors controlling large regulons. (Supplementary Figure 10 and Supplementary Information)

The PARADIGM-inferred pathway signatures were further used to derive a map of the genetic mechanisms that may underlie treatment response. A subnetwork was constructed in which interactions were retained only if they connected two features with higher than average absolute association with Ki67 biomarker status. (Supplementary Figure 10,
Figure 6.4: Key cancer pathway components altered in luminal breast tumours. (A) Only genetic alterations identified in 46 WGS cases are shown. Alterations were discovered in key genes in the TP53/RB, MAPK, PI3K/AKT/mTOR pathways. Genes coloured blue and red are predicted to be functionally inactivated and activated, respectively, through focused mutations including point mutations and small indels (M), copy number deletions (C), or other structural changes (S) that affect the gene. The inter-connectedness of this network (several pathways) shows that there are many different ways to perturb a pathway.

continued on next page
(B) Eight interaction networks from canonical maps are significantly over-represented by mutations in 77 luminal breast tumours (46 WGS and 31 exome cases). In the concentric circle diagram, tumours are arranged as radial spokes and categorized by their mutation status in each network (concentric ring colour) and SMG mutation status (black dots). Tumour classification by pathway analysis shows many tumours unaffected by a given SMG often harbour other mutations in the same network. For full annotation, see Supplementary Information and Supplementary Figure 6.6. PLC: phospholipase C.
Supplementary Figure 11, and Supplementary Information) Consistent with the PathScan results, among the largest of the hubs in the identified network were a central DNA damage hub with the second highest connectivity (55 regulatory interactions; 1% of the network) and \textit{TP53} with the 14\textsuperscript{th} highest connectivity (26 connections; 0.5% of the network). Additional highly connected hubs identified in order of connectivity were \textit{MYC} with 79 connections (1.4\%), \textit{FYN} with 45 (0.8\%), \textit{MAPK3} with 43, \textit{JUN} with 40, \textit{HDAC1} with 40, \textit{SHC1} with 39, and \textit{HIF1A}/\textit{ARNT} complex with 39. (Supplementary Figure 11)

To identify higher-level connections between mutations and clinical features, we compared the samples on the basis of pathway-derived signatures. For each clinical attribute and each SMG, we dichotomized the discovery samples into a positive and a negative group to derive pathway signatures that discriminated between the groups. (see details in Supplementary Information) We then computed all pair-wise Pearson correlations between pathway signatures and clustered the resulting correlations. (Figure 6.5) The entire process was repeated using validated mutations and signatures derived from the validation set. (Supplementary Figure 12) In line with expectation, \textit{PIK3CA}, \textit{MAP3K1}, \textit{MAP2K4}, and low risk preoperative endocrine prognostic index (PEPI) scores (PEPI is an index of recurrence risk post neoadjuvant aromatase inhibitor therapy) cluster with the luminal A subtypes and with each other, and are supported by the validation set analysis.\textsuperscript{4} The luminal B-like signatures included \textit{TP53}, \textit{RB1}, \textit{RUNX1} and \textit{MALAT1}, which also associated with other poor outcome features such as high baseline and surgical Ki67 levels, high grade histology and high PEPI scores. The \textit{TP53} and \textit{MALAT1} associations in the discovery set also were supported by the validation set analysis.
Figure 6.5: Pathway signatures reveal connections between mutations and clinical outcomes. PARADIGM-based pathway signatures were derived for tumour feature dichotomies including mutation driven gene signatures (mutant versus non-mutant), histopathology type (lobular versus ductal), PEPI score (PEPI = 0 favourable versus PEPI > 0 unfavourable), PAM50 (50-gene intrinsic breast cancer subtype classifier) luminal A subtype (luminal A versus luminal B) and the reverse (luminal B versus luminal A), histopathology grade (grades II and III versus I), baseline Ki67 levels (≥14% versus <14%), and end-of-treatment Ki67 levels (≥10% versus <10%) and overall PEPI score (higher than mean unfavourable versus lower than mean favourable). Pearson correlations were computed between all pair-wise signatures; positive correlations, red; negative correlations, blue; column features ordered identically as rows. Correlation analysis on the 77 samples in the discovery set is shown. Asterisk: Ki67 < 2.7%, oestrogen-receptor-positive, node negative, and tumour size ≤ 5 cm.
Druggable gene analysis

We defined mutations in druggable tyrosine kinase domains including in \textit{ERBB2} (a V777L and a 755–759\textsuperscript{LRENT} in-frame deletion homologous to gefitinib-sensitizing \textit{EGFR} mutations in lung cancer), as well as in \textit{DDR1} (A829V, R611C), \textit{DDR2} (E583D), \textit{CSF1R} (D735H, M875L), and \textit{PDGFRA} (E924K). In addition, pleckstrin homology domain mutations were observed in \textit{AKT1} (C77F) and \textit{AKT2} (S11F) and a kinase domain mutation was identified in \textit{RPS6KB1} (S375F). (Supplementary Table 18)

6.2.4 Discussion

The low frequency of many SMGs presents an enormous challenge for correlative analysis, but several statistically significant patterns were identified, including the relationship between \textit{MAP3K1} mutation, luminal A subtype, low tumour grade and low Ki67 proliferation index. On this basis, for patients with \textit{MAP3K1} mutant luminal tumours, neoadjuvant aromatase inhibitor could provide a favourable option. In contrast, tumours with \textit{TP53} mutations, which are mostly aromatase inhibitor resistant, would be more appropriately treated with other modalities. \textit{MAP3K1} activates the ERK family, thus, loss of ERK signalling could explain the indolent nature of \textit{MAP3K1}-deficient tumours. However, \textit{MAP3K1} also activates JNK through \textit{MAP2K4}, which also can be mutated. Loss of JNK signalling produces a defect in apoptosis in response to stress, which would hypothetically explain why these mutations accumulate.\textsuperscript{41,42} \textit{PIK3CA} harboured the most mutations (41.3%) but was neither associated with clinical nor Ki67 response, confirming our earlier report.\textsuperscript{43} However, the positive association between \textit{MAP3K1/MAP2K4} mutations and \textit{PIK3CA} mutation at both the mutation and pathway levels suggests cooperativity. (Figure 6.4A)
The finding of multiple SMGs linked previously to benign and malignant haematopoietic disorders suggests that breast cancer, like leukaemia, can be viewed as a stem-cell disorder that produces indolent or aggressive tumours that display varying phenotypes depending on differentiation blocks generated by different mutation repertoires. Whereas only \textit{MLL3} showed statistical significance in the analysis of 46 WGS cases, multiple mutations in genes related to histone modification and chromatin remodelling are worth noting. (Supplementary Table 19) An array of coding mutations and structural variations was discovered in methyltransferases (\textit{MLL2, MLL3, MLL4 and MLL5}), demethyltransferases (\textit{KDM6A, KDM4A, KDM5B and KDM5C}), and acetyltransferases (\textit{MYST1, MYST3 and MYST4}). Furthermore, our analysis identified several adenine-thymine (AT)-rich interactive domain-containing protein genes (\textit{ARID1A, ARID2, ARID3B and ARID4B}) that harboured mutations and large deletions, reinforcing the role of members from the SNF/SWI family in breast cancer.

Pathway analysis enables the evaluation of mutations with low recurrence frequency where statistical comparisons are conventionally underpowered. For example, the eight samples with \textit{MAP2K4} mutations were sufficient to derive a reliable pathway-based gene signature in PARADIGM that aligns with \textit{MAP3K1}. This approach also pointed to a putative connection between \textit{MALAT1} and the \textit{TP53} pathway. Finally, we provide evidence that transcriptional associations to Ki67 response reside in a connected network under the control of several key ‘hub’ genes including \textit{MYC, FYN} and \textit{MAP} kinases, among others. Targeting these hubs in resistant tumours could produce therapeutic advances. In conclusion, the genomic information derived from unbiased sequencing is a logical new starting point for clinical investigation, where the mutation status of an individual patient is determined in advance and treatment decisions are driven by therapeutic hypotheses that stem from knowledge of
the genomic sequence and its possible consequences. However, the accrual of large numbers of patients and the use of comprehensive sequencing and gene expression approaches will be required because of the extreme genomic heterogeneity documented by this investigation.

6.2.5 Methods Summary

Clinical trial samples were accessed from the preoperative letrozole phase 2 study (NCT00084396) that investigated the effect of letrozole for 16 to 24 weeks on surgical outcomes and from the American College of Surgeons Oncology Group (ACOSOG) Z1031 study (NCT00265759) that compared anastrozole with exemestane or letrozole for 16 to 18 weeks before surgery. Baseline snap-frozen biopsy samples with greater than 70% tumour content (by nuclei) underwent DNA extraction and were paired with a peripheral blood DNA sample. Two formalin-fixed biopsies were obtained at baseline and at surgery, and were used to conduct oestrogen receptor and Ki67 immunohistochemistry as previously published. Paired end Illumina reads from tumours and normal samples were aligned to NCBI build36 using BWA. Somatic point mutations were identified using SomaticSniper, and indels were identified by combining results from a modified version of the Samtools indel caller (http://samtools.sourceforge.net/), GATK and Pindel. Structural variations were identified using BreakDancer and SquareDancer (unpublished). All putative somatic events found in 46 cases were validated by targeted custom capture arrays (Nimblegen)/Illumina sequencing and all tier 1 mutations for 46 WGS cases also were validated using PCR/454 sequencing. All statistical analyses, including SMG, mutation relation and clinical correlation were done using the MuSiC package and/or by standard statistical tests. (Supplementary Information) Pathway analysis was performed with PathScan, GeneGo Metacore (http://www.genego.com/metacore.php) and
PARADIGM. A complete description of the materials and methods used to generate this data set and results is provided in the Supplementary Methods section.
Supplementary Figure 6.6: Key cancer pathway components altered in luminal breast tumours. 

*continued on next page*
Eight interaction networks from canonical maps are significantly over-represented by mutations in 77 luminal breast tumours (46 WGS and 31 exome cases). Maps were grouped by function and arranged to reflect overall hierarchy of signaling through protein-protein interactions. Mutated genes are represented in black and sized to reflect the number of tumors containing each mutation. SMGs are shown as red nodes with reference numbers indicated in the key. White nodes are non-mutated network objects. In the concentric circle diagram, tumors are arranged as radial spokes and categorized by their mutation status in each network (concentric ring color) and SMG mutation status (black dots). Tumor classification by pathway analysis shows many tumors unaffected by a given SMG often harbor other mutations in the same network. For full annotation, see Supplementary Information for Gene Interaction Network Analysis.
6.2.6 Supplementary methods

**Gene Interaction Network Analysis**  The interaction networks were compiled from Metacore maps (GeneGo, Inc.) significantly intersecting with the mutation list (corrected FDR < 0.05). Mutated genes in multiple network objects were only considered in one object to prevent redundant mutation counts. Network objects were classified into eight functional networks and arranged hierarchically in Cytoscape.\(^{46,47}\) SMGs \textit{AGTR2}, \textit{STMN2}, \textit{MLL3}, and \textit{SF3B1} were not included in any maps by Metacore, so curated interactions were added with members of the most applicable network. Some interactions between non-mutated nodes were removed for clarity. The number of tumors harboring a node mutation is indicated by node size. SMGs from each network are differentiated in red and by a reference number; other mutated objects are colored black. All 77 tumors were classified by network and SMG mutation status in a concentric display. Each patient is represented by a spoke in the wheel, affected networks for each patient are indicated by the appropriate color, and SMG mutations are shown as black dots in the parent network. The MLL2/3 complex is represented as a single network node with multiple mutated constituent genes. Of these, only \textit{MLL3} was considered an SMG in patient classification. (Supplementary Data Files)

6.3 References


Manuscript abbreviations

FDR False discovery rate
indel Insertion/deletion
Mb Megabase
NHEJ Non-homologous end joining
PEPI Preoperative endocrine prognostic index
SMG Significantly mutated gene
SNV Single nucleotide variant
WGS Whole-genome sequencing
Chapter 7

Statistical and Computational Analysis

Methods

7.1 Introduction

Various computational tools were developed throughout the work presented herein. Some tools are limited to macros or simple programs to assist in the imaging data analysis pipeline, while others are new statistical methods adapted from peer-reviewed techniques developed with accompanying programming to automate complex analyses. This chapter contains both source code and step-by-step explanation of new statistical techniques that generalizable to broad applications; specific notes on uses particular to the aforementioned experiments appear as footnotes where appropriate.

7.2 WLS calibration algorithm

Statistical basis

Throughout this project, data were analyzed with a weighted least squares (WLS) regression algorithm adapted from multiple literature reports on calibration methods. The method
constructs a standard curve by weighting each calibration standard according to the inter-replicate variance and comparing first- and second-order models on the basis of degrees of freedom and regression residuals. In the detailed description of the method that follows, Table 7.1 may be used as a reference for the parameters found throughout.

This method uses the standard General Linear Model assumption that the error in independent variable $x$ is negligible compared to that of the dependent measurements $y$. Standard concentrations $x_j$ ($j = 1, 2, \ldots, k$) in the calibration curve are each prepared as technical replicates $i$ with $i_j = 1, 2, \ldots, m_j$ and giving the total number of points used in calibration $n = \sum_{j=1}^{k} m_j$.\(^1\) The standard deviation $s_j$ of measurements $y_{ij}$ is used in the regression equation (7.1) to determine the parameter $\theta$, which is then used to calculate an estimated standard deviation under the assumption that $\sigma \propto y$:\(^2\)

$$s = \hat{\theta} \bar{y} \rightarrow \theta \bar{y}_j = \bar{s}_j$$

(7.1)

These estimated\(^3\) standard deviations are used to weight\(^4\) each calibration point $j$:

$$w_j = \frac{1}{\bar{s}_j^2} = \frac{1}{(\theta \bar{y}_j)^2}$$

(7.2)

Using the weights assigned to each calibration standard, the WLS algorithm next computes first- and second-order models for the data. The quadratic WLS regression curve is estimated in the form

$$\bar{y}_{[Q]} = \bar{y}_w + b_1 (x - \bar{x}_w) + b_2 (x^2 - \bar{x}_w^2)$$

(7.3)

\(^1\)In all experiments presented in Chapter 4, $m_j = 3$ for all $1 \leq j \leq 8$; thus $n = 24$.

\(^2\)This method has a key advantage over estimating $\bar{s}_j$ using $x_j$: it prevents $\bar{s}_j = 0$ so long as $y (0, \infty]$ and thus prevents undefined weights $w_{x=0}$ as well as preventing outliers at high $x_j$ from artificially shifting the weighted mean $\bar{x}_w$.

\(^3\)The WLS algorithm can optionally be set to use measured $s_j$ to determine weights, but $\bar{s}_j$ is still required for inverse regression so this behavior is disabled by default.

\(^4\)Should any values of $\bar{s}_j$ be sufficiently small to cause $w_j = 0$ due to float point precision errors, the algorithm scales all values of $\bar{s}_j$ by the mean of all $\bar{s}$ to maintain relative weighting and recomputes all $w_j$. 

[203]
where $\hat{y}_{[Q]}$ is the mean regression estimate as a function of $x$,

$$
\bar{y}_w = \frac{\sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j y_{ij}}{\sum_{j=1}^{k} m_j w_j} \tag{7.4}
$$

is the weighted mean of all calibration measurements,

$$
\bar{x}_w = \frac{\sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j x_{ij}}{\sum_{j=1}^{k} m_j w_j} \tag{7.5}
$$

is the weighted mean of all calibration standard values, and

$$
\bar{x^2}_w = \frac{\sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j x^2_{ij}}{\sum_{j=1}^{k} m_j w_j} \tag{7.6}
$$

is the weighted mean of all squared calibration standard values. To calculated the remaining parameters $b_1$ and $b_2$, the minimum sum of the squared residuals is computed by partially differentiating $SSE$ in terms of each unknown parameter, setting each $\frac{\partial SSE}{\partial b} = 0$, and solving the resulting system of equations for the unknown parameters.

$$
SSE = \sum_{j=1}^{k} \sum_{i=1}^{m_j} (y_{ij} - \hat{y}_{ij[Q]})^2 \tag{7.7}
$$
To make the equations for $b_1$ and $b_2$ more compact, some intermediate variables are defined:

\[
\Sigma_{x^2} = \sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j x_{ij}^2 - \left( \sum_{j=1}^{k} m_j w_j \right) (\bar{x}_w)^2 \quad (7.8)
\]

\[
\Sigma_{xy} = \sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j x_{ij} y_{ij} - \left( \sum_{j=1}^{k} m_j w_j \right) \bar{x}_w \bar{y}_w \quad (7.9)
\]

\[
\Sigma_{xs} = \sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j x_{ij}^3 - \left( \sum_{j=1}^{k} m_j w_j \right) \bar{x}_w x_w^2 \quad (7.10)
\]

\[
\Sigma_{sy} = \sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j x_{ij}^2 y_{ij} - \left( \sum_{j=1}^{k} m_j w_j \right) \bar{x}_w^2 y_w \quad (7.11)
\]

\[
\Sigma_{s^2} = \sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j x_{ij}^4 - \left( \sum_{j=1}^{k} m_j w_j \right) (\bar{x}_w^2)^2 \quad (7.12)
\]

\[
\Sigma_{D[Q]} = \Sigma_{x^2} \Sigma_{s^2} - (\Sigma_{xs})^2 \quad (7.13)
\]

From these, values for $b_1$ and $b_2$ are computed by

\[
b_1 = \frac{\Sigma_{xy} \Sigma_{s^2} - \Sigma_{xs} \Sigma_{sy}}{\Sigma_{D[Q]}} \quad (7.14)
\]

\[
b_2 = \frac{\Sigma_{x^2} \Sigma_{sy} - \Sigma_{xs} \Sigma_{xy}}{\Sigma_{D[Q]}} \quad (7.15)
\]

These are then used in equation (7.3) to find $\bar{y}_{j[Q]}$ so that the weighted residual standard deviation can be computed by

\[
s_{Rw[Q]} = \sqrt{\frac{k \sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j \left( y_{ij} - \bar{y}_{j[Q]} \right)^2}{n - 3}} \quad (7.16)
\]

The linear WLS model is then similarly computed for comparison with the quadratic. The model takes a familiar form of equation (7.3) with $b_2 = 0$ the differentiation strategy
described for quadratic regression is simplified:

\[
\tilde{y}_{[L]} = \bar{y}_w + b_1 (x - \bar{x}_w)
\]  

(7.17)

\[
\Sigma_{D[L]} = \sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j (x_{ij} - \bar{x}_w)^2
\]  

(7.18)

\[
b_1 = \frac{\sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j (x_{ij} - \bar{x}_w) y_{ij}}{\Sigma_{D[L]}} \quad b_2 = 0
\]  

(7.19)

\[
s_{Rw[L]} = \sqrt{\frac{\sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j \left(y_{ij} - \tilde{y}_{j[L]}\right)^2}{n - 2}}
\]  

(7.20)

The two models are compared using the \(F\)-ratio test with the critical value \(F_{\alpha/2}^{1,n-3}\) determined by the desired false positive rate \(\alpha\):

\[
F_{\text{test}} = \frac{\left(\frac{\sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j \left(y_{ij} - \tilde{y}_{j[L]}\right)^2 - \sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j \left(y_{ij} - \tilde{y}_{j[Q]}\right)^2}{n - 3}\right)}{\sum_{j=1}^{k} \sum_{i=1}^{m_j} w_j \left(y_{ij} - \tilde{y}_{j[Q]}\right)^2}
\]  

(7.21)

The quadratic WLS model is used only if \(F_{\text{test}} > F_{\alpha/2}^{1,n-3}\).

Once the appropriate model is selected, the results of the regression are plotted with equation (7.3) or (7.17) and solved algebraically to find estimated values \(x_u\) for each unknown sample \(u\). For linear,

\[
x_{u[L]} = \bar{x}_w - \frac{\bar{y}_u - \bar{y}_w}{b_1}
\]  

(7.22)

and for quadratic,

\[
x_{u[Q]} = \begin{cases} 
\frac{-b_1 - \sqrt{4b_2 (\bar{y}_u - \bar{y}_w) + 4b_2^2 \bar{x}_w^2 + b_1^2 + 4b_1 b_2 \bar{x}_w}}{2b_2} & \text{if } b_1 < 0 \\
\frac{-b_1 + \sqrt{4b_2 (\bar{y}_u - \bar{y}_w) + 4b_2^2 \bar{x}_w^2 + b_1^2 + 4b_1 b_2 \bar{x}_w}}{2b_2} & \text{if } b_1 > 0 
\end{cases}
\]  

(7.23)
WLS calibration algorithm

Two band intervals can be predicted about the regression model. To simplify notation, the term $K$ is defined for both linear and quadratic models:

\[
K[L] = \frac{1}{\sum_{j=1}^{k} m_j w_j} + \frac{(x - \bar{x}_w)^2}{\sum D[L]} 
\]

\[
K[Q] = \frac{1}{\sum_{j=1}^{k} m_j w_j} + \frac{(x - \bar{x}_w)^2 \Sigma s^2}{\sum D[Q]} + \frac{(x^2 - \bar{x}_w^2)^2 \Sigma s^2}{\sum D[Q]} - 2 \frac{(x - \bar{x}_w)(x^2 - \bar{x}_w^2) \Sigma x}{\sum D[Q]} 
\] (7.25)

The confidence bands can be thought of as the confidence intervals for the calibration curve itself:

\[
\hat{y}_{\text{conf}[L]} = \bar{y}[L] \pm s_{Rw[L]} t_{1-\alpha/2,n-2} \sqrt{K[L]} 
\]

\[
\hat{y}_{\text{conf}[Q]} = \bar{y}[Q] \pm s_{Rw[Q]} t_{1-\alpha/2,n-3} \sqrt{K[Q]} 
\] (7.27)

where $t_{1-\alpha/2,n-3}$ and $t_{1-\alpha/2,n-2}$ are the critical values of the $t$-distribution with a false positive error rate $\alpha$ and degrees of freedom accounting for quadratic and linear regression parameters, respectively. Prediction bands give the confidence intervals for the mean of $m$ replicate measurements on the measurement uncertainty implied by the regression variance.\(^5\)

\[
\hat{y}_{\text{pred}[L]} = \bar{y}[L] \pm s_{Rw[L]} t_{1-\alpha/2,n-2} \sqrt{\frac{(\bar{y}_w + b_1 (x - \bar{x}_w))^2 \theta^2}{m} + K[L]} 
\]

\[
\hat{y}_{\text{pred}[Q]} = \bar{y}[Q] \pm s_{Rw[Q]} t_{1-\alpha/2,n-3} \sqrt{\frac{(\bar{y}_w + b_1 (x - \bar{x}_w) + b_2 (x^2 - \bar{x}_w^2))^2 \theta^2}{m} + K[Q]} 
\] (7.29)

These prediction bands can be used to find the minimum a priori detectable concentration of the analyte, $\hat{x}_\lambda$, based on the false positive error rate $\alpha$, the false negative error rate $\beta$, and the number of technical replicates $m$. First, the critical signal $y_\kappa$ associated with the one-sided $t$-distribution expected for $\hat{y}_{x=0}$ is computed with certainty $\alpha$:

\[
y_\kappa[L] = \bar{y}_w - b_1 \bar{x}_w + s_{Rw[L]} t_{1-\alpha,n-2} \sqrt{\frac{(\bar{y}_w - b_1 \bar{x}_w)^2 \theta^2}{m}} + \frac{1}{\sum_{j=1}^{k} m_j w_j} + \frac{\bar{x}_w^2}{\sum D[L]} 
\] (7.30)

\(^5\)the confidence bands are a special case of the prediction bands with $m \to \infty$
\[ y_{\kappa}[Q] = \bar{y}_w - b_1 \bar{x}_w - b_2 \bar{x}_w^2 \]

\( + s_{Rw[Q]} t_{1-\alpha,n-3} \frac{\left( \bar{y}_w - b_1 \bar{x}_w - b_2 \bar{x}_w^2 \right)^2 \theta^2}{m} + \frac{1}{\sum_{j=1}^{k} m_j w_j} + \frac{\bar{x}_w^2 \Sigma s^2 + (\bar{x}_w^2)^2 \Sigma x^2 - 2 \bar{x}_w x^2 \Sigma x}{\Sigma D[Q]} \)  

(7.31)

The minimum expected analyte \( \bar{x}_\lambda \) detectable with power \( 1 - \beta \) is found by solving for its expected \( t \)-distribution with the critical value \( \beta \) intersecting with \( y_\kappa \):

\[ y_{\kappa}[L] = \bar{y}_w + b_1 \left( \bar{x}_\lambda[L] - \bar{x}_w \right) \]

\( + s_{Rw[L]} t_{\beta,n-2} \frac{\left( \bar{y}_w + b_1 \left( \bar{x}_\lambda[L] - \bar{x}_w \right) \right)^2 \theta^2}{m} + \frac{1}{\sum_{j=1}^{k} m_j w_j} + \frac{\bar{x}_\lambda[L] - \bar{x}_w}{\Sigma D[L]} \)  

(7.32)

\[ y_{\kappa}[Q] = \bar{y}_w + b_1 \left( \bar{x}_\lambda[Q] - \bar{x}_w \right) + b_2 \left( \bar{x}_\lambda[Q] - \bar{x}_w^2 \right) \]

\( + s_{Rw[Q]} t_{\beta,n-3} \left( \frac{\left( \bar{y}_w + b_1 \left( \bar{x}_\lambda[Q] - \bar{x}_w \right) + b_2 \left( \bar{x}_\lambda[Q] - \bar{x}_w^2 \right) \right)^2 \theta^2}{m} + \frac{1}{\sum_{j=1}^{k} m_j w_j} + \left( \frac{\bar{x}_\lambda[Q] - \bar{x}_w}{\Sigma D[Q]} \right)^2 + \left( \frac{\bar{x}_\lambda[Q] - \bar{x}_w^2}{\Sigma D[Q]} \right)^2 \Sigma x^2 - 2 \bar{x}_w x^2 \Sigma x \right)^{\frac{1}{2}} \)  

(7.33)

Equations (7.32) and (7.33) are not analytically solvable and are thus solved iteratively for \( \bar{x}_\lambda \) without limits of precision.

Although the prediction bands are sufficient for single comparisons and graphical representation of calibration curves, they do not correct for true error rate resulting from repeated use of the same standards. To correct for this, a third interval for inverse regression
WLS calibration algorithm is computed by using the $F$-distribution.\textsuperscript{5}

\begin{align*}
\bar{y}_{\text{cal}[L]} &= \bar{y}_{[L]} \pm s_{Rw[L]} \sqrt{2F_{2,n-2}^{\alpha/2} K_{[L]}} \\
\bar{y}_{\text{cal}[Q]} &= \bar{y}_{[Q]} \pm s_{Rw[Q]} \sqrt{3F_{3,n-3}^{\alpha/2} K_{[Q]}}
\end{align*}

(7.34) \quad (7.35)

These equations demonstrate the trade-off required to use the more-accurate quadratic model: as $2F_{2,n-2}^{\alpha/2} < 3F_{3,n-3}^{\alpha/2}$ is always true, the quadratic model is more precise than the linear only when this inequality is compensated for by the relationship between $s_{Rw[Q]}$ and $s_{Rw[L]}$.

The intersection of this band with the $t$-distribution of $m_u$ unknown sample replicates with mean $\bar{y}_u$ and standard deviation $s_u$ determines the confidence intervals about the calibration result. The upper and lower bounds for replicates of an unknown sample calibrated with a linear curve are found by solving the following equations for $\bar{x}_{u,\text{min}[L]}$ and $\bar{x}_{u,\text{max}[L]}$:

\begin{align*}
\bar{y}_u - \frac{s_u}{\sqrt{m_u}} f_{1-\alpha/2,m_u-1} &= \bar{y}_w + b_1 \left( \bar{x}_{u,\text{min}[L]} - \bar{x}_w \right) \\
&\quad + s_{Rw[L]} \sqrt{2F_{2,n-2}^{\alpha/2}} \sqrt{\frac{1}{\sum_{j=1}^{k} m_j w_j} + \frac{(\bar{x}_{u,\text{min}[L]} - \bar{x}_w)^2}{\sum D[L]}} \tag{7.36}
\end{align*}

\begin{align*}
\bar{y}_u + \frac{s_u}{\sqrt{m_u}} f_{1-\alpha/2,m_u-1} &= \bar{y}_w + b_1 \left( \bar{x}_{u,\text{max}[L]} - \bar{x}_w \right) \\
&\quad - s_{Rw[L]} \sqrt{2F_{2,n-2}^{\alpha/2}} \sqrt{\frac{1}{\sum_{j=1}^{k} m_j w_j} + \frac{(\bar{x}_{u,\text{max}[L]} - \bar{x}_w)^2}{\sum D[L]}} \tag{7.37}
\end{align*}
The quadratic model equations are analogously solved for \( \bar{x}_{u,\text{min}[Q]} \) and \( \bar{x}_{u,\text{max}[Q]} \):

\[
\bar{y}_u - \frac{s_u}{\sqrt{m_u}} t_{1-\alpha/2,m_u-1} = \bar{y}_w + b_1 \left( \bar{x}_{u,\text{min}[Q]} - \bar{x}_w \right) + b_2 \left( \bar{x}_{u,\text{min}[Q]} - \bar{x}_w^2 \right)
+ s_{Rw[Q]} \sqrt{3 \frac{F_{\alpha/2}}{3,n-3}} \left( \sum_{j=1}^{k} \frac{1}{m_j w_j} \left( \frac{\bar{x}_{u,\text{min}[Q]} - \bar{x}_w}{\Sigma_{D[Q]}} \right)^2 \Sigma s^2 \frac{\bar{x}_{u,\text{min}[Q]} - \bar{x}_w}{\Sigma_{D[Q]}} \right)^{\frac{1}{2}}
\]

\[
\bar{y}_u + \frac{s_u}{\sqrt{m_u}} t_{1-\alpha/2,m_u-1} = \bar{y}_w + b_1 \left( \bar{x}_{u,\text{max}[Q]} - \bar{x}_w \right) + b_2 \left( \bar{x}_{u,\text{max}[Q]} - \bar{x}_w^2 \right)
- s_{Rw[Q]} \sqrt{3 \frac{F_{\alpha/2}}{3,n-3}} \left( \sum_{j=1}^{k} \frac{1}{m_j w_j} \left( \frac{\bar{x}_{u,\text{max}[Q]} - \bar{x}_w}{\Sigma_{D[Q]}} \right)^2 \Sigma s^2 \frac{\bar{x}_{u,\text{max}[Q]} - \bar{x}_w}{\Sigma_{D[Q]}} \right)^{\frac{1}{2}}
\]

The full expansions of equations (7.38) and (7.39) demonstrate why \( x_{u,\text{min}} \) and \( x_{u,\text{max}} \) must be solved iteratively for numeric solutions rather than analytically.

**Implementation**

The code for WLS calibration was written in SPSS version 19, but has been updated most recently using SPSS 21. No new functions were introduced during the rewrites, but other SPSS versions have not been tested. The program requires a minimum of 4 SPSS variables indicating the following quantities:

1. **measurement**: numeric variable (absorbance, luminescence, etc.)
Table 7.1: Parameters used in WLS regression.

<table>
<thead>
<tr>
<th>parameter(s)</th>
<th>description</th>
<th>equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$x_j$</td>
<td>value of the regression independent variable (concentration)</td>
<td>–</td>
</tr>
<tr>
<td>$y_{ij}$</td>
<td>measurement of the dependent variable at $x = x_j$</td>
<td>–</td>
</tr>
<tr>
<td>$j, k$</td>
<td>calibration concentrations with $j = 1, 2, \ldots, k$</td>
<td>–</td>
</tr>
<tr>
<td>$i_j, m_j$</td>
<td>replications of each standard calibration point $x_j$ with $i_j = 1, 2, \ldots, m_j$</td>
<td>–</td>
</tr>
<tr>
<td>$n$</td>
<td>total calibration measurements, $\sum_{j=1}^k m_j$</td>
<td>–</td>
</tr>
<tr>
<td>$s_j, \sigma_j$</td>
<td>the standard deviation of $y_j$ and the theoretical $\lim_{m_j \to \infty} s_j$</td>
<td>(7.1)</td>
</tr>
<tr>
<td>$\theta$</td>
<td>regression term used to find $\tilde{s}_j$</td>
<td>(7.1)</td>
</tr>
<tr>
<td>$\tilde{s}_j$</td>
<td>estimated standard deviation for $y_j$</td>
<td>(7.1)</td>
</tr>
<tr>
<td>$\tilde{y}_j$</td>
<td>the unweighted mean of $y_j$</td>
<td>–</td>
</tr>
<tr>
<td>$w_j$</td>
<td>weight of calibration point $j$</td>
<td>(7.2)</td>
</tr>
<tr>
<td>$\tilde{y}_{[Q]}$</td>
<td>measurement at $x$ predicted by quadratic WLS regression</td>
<td>(7.3)</td>
</tr>
<tr>
<td>$\tilde{y}_{[L]}$</td>
<td>measurement at $x$ predicted by linear WLS regression</td>
<td>(7.17)</td>
</tr>
<tr>
<td>$\bar{y}_j$</td>
<td>weighted mean of all calibration measurements, also the $0^{th}$ order regression term</td>
<td>(7.4)</td>
</tr>
<tr>
<td>$b_1$</td>
<td>first-order regression term</td>
<td>(7.14), (7.19)</td>
</tr>
<tr>
<td>$b_2$</td>
<td>second-order regression term</td>
<td>(7.15)</td>
</tr>
<tr>
<td>$\bar{x}_w$</td>
<td>weighted mean of all calibration standard values</td>
<td>(7.5)</td>
</tr>
<tr>
<td>$\bar{x}_{w}^2$</td>
<td>weighted mean of all squared calibration standard values</td>
<td>(7.6)</td>
</tr>
<tr>
<td>$\Sigma_{x^2}$</td>
<td>Intermediate computation terms</td>
<td>–</td>
</tr>
<tr>
<td>$\Sigma_{xy}$</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>$\Sigma_{x}^2$</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>$\Sigma_{ab}$</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>$\Sigma_{b}$</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>$\Sigma_{D[Q]}$</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>$\Sigma_{D[L]}$</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>$K_{[L]}$</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>$K_{[Q]}$</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>the false positive error rate, also known as the critical $p$ value</td>
<td>–</td>
</tr>
<tr>
<td>$\beta$</td>
<td>the false negative error rate, $1 – power$</td>
<td>–</td>
</tr>
<tr>
<td>$t_{\alpha/\tau, \delta}$</td>
<td>7-sided $t$-distribution critical value with probability $\alpha$ and $\delta$ degrees of freedom</td>
<td>–</td>
</tr>
<tr>
<td>$F_{\alpha/\tau, \delta, \epsilon}$</td>
<td>7-sided $F$-distribution critical value with probability $\alpha$ and $\delta, \epsilon$ degrees of freedom</td>
<td>–</td>
</tr>
<tr>
<td>$F_{test}$</td>
<td>$F$-distribution value used to compare linear and quadratic WLS regression</td>
<td>(7.21)</td>
</tr>
<tr>
<td>$s_{Rw[Q]}$</td>
<td>standard deviation of quadratic regression residuals</td>
<td>(7.16)</td>
</tr>
<tr>
<td>$s_{Rw[L]}$</td>
<td>standard deviation of linear regression residuals</td>
<td>(7.20)</td>
</tr>
<tr>
<td>$\tilde{y}_{conf[L]}$</td>
<td>confidence and prediction intervals for linear and quadratic WLS regression</td>
<td>(7.26)</td>
</tr>
<tr>
<td>$\tilde{y}_{conf[Q]}$</td>
<td></td>
<td>(7.27)</td>
</tr>
<tr>
<td>$\tilde{y}_{pred[L]}$</td>
<td></td>
<td>(7.28)</td>
</tr>
<tr>
<td>$\tilde{y}_{pred[Q]}$</td>
<td></td>
<td>(7.29)</td>
</tr>
<tr>
<td>$\bar{y}_{[L]}$</td>
<td>critical $\tilde{y}$ indicating non-zero analyte with confidence $1 – \alpha$</td>
<td>(7.30)</td>
</tr>
<tr>
<td>$\bar{y}_{[Q]}$</td>
<td></td>
<td>(7.31)</td>
</tr>
<tr>
<td>$\bar{x}_{[L]}$</td>
<td>minimal detectable analyte with false positive rate $\alpha$ and false negative rate $\beta$</td>
<td>(7.32)</td>
</tr>
<tr>
<td>$\bar{x}_{[Q]}$</td>
<td></td>
<td>(7.33)</td>
</tr>
<tr>
<td>$\tilde{y}_{cal[L]}$</td>
<td>multiple-comparison calibration intervals to determine unknown analyte from repeated measures</td>
<td>(7.34)</td>
</tr>
<tr>
<td>$\tilde{y}_{cal[Q]}$</td>
<td></td>
<td>(7.35)</td>
</tr>
<tr>
<td>$\bar{x}_{u[L]}$</td>
<td>Inverse regression results with upper and lower bounds for unknown sample $u$</td>
<td>(7.22)</td>
</tr>
<tr>
<td>$\bar{x}_{u[Q]}$</td>
<td></td>
<td>(7.23)</td>
</tr>
<tr>
<td>$\bar{x}_{umin[L]}$</td>
<td></td>
<td>(7.36)</td>
</tr>
<tr>
<td>$\bar{x}_{umax[L]}$</td>
<td></td>
<td>(7.37)</td>
</tr>
<tr>
<td>$\bar{x}_{umin[Q]}$</td>
<td></td>
<td>(7.38)</td>
</tr>
<tr>
<td>$\bar{x}_{umax[Q]}$</td>
<td></td>
<td>(7.39)</td>
</tr>
</tbody>
</table>
Table 7.2: Example of minimal dataset fitting the requirements of the WLS regression program for SPSS. Unknown samples are not strictly required; calibration curves can be constructed without being used to determine unknown analyte concentrations. Note that the data file must contain at least 4 rows to satisfy the required conditions, 6 rows if there are any unknowns being regressed.

<table>
<thead>
<tr>
<th>Analyte measurement group known</th>
<th>Known</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3713</td>
<td>2</td>
</tr>
<tr>
<td>4175</td>
<td>2</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
</tr>
</tbody>
</table>

2. **analyte concentrations**: numeric variable, the known standard curve values (does not need to be blank for “unknown” concentrations) must have at least 2 duplicates per value

3. **standard indicator**: binary variable, 1 for all values of the standard curve, 0 for all other measurements to be estimated by the standards

4. **group**: no format requirement, must be unique for every set of measurements

In addition to the standard SPSS variable naming rules, no variables may be named “x” or “y” for programming reasons. An example of data meeting the minimum requirements can be found in Table 7.2. Running the WLS regression requires two steps: first, the program must be called, either by running the code directly or by calling the program with the `insert file=` command as shown below in code block 7.2.

Source Code 7.1: WLS regression

```bash
1 DEFINE !WLSreg1 ( stdVals=!tokens(1) / grpVar=!tokens(1) )
```

For example, an experiment with 3 standard concentrations and 2 unknown samples requires 5 unique values of this grouping variable

[212]
WLS calibration algorithm

* set output to prevent clutter in analysis window
SET PRINTBACK = NONE RESULTS = NONE.
OUTPUT NAME mainOutput.
OUTPUT NEW.
OUTPUT NAME dummyOutput.
OUTPUT ACTIVATE dummyoutput.

* prepare standard variable names for convenient coding.
DATASET NAME calibration.
AUTORECODE !grpVar / INTO Split.
VALUE LABELS split.
IF !stdGrp x = !stdVals.
IF !stdGrp y = !Mxs.
EXECUTE.

* find calibration replicate coefficients of variation.
AGGREGATE
/OUTFILE=* MODE = ADDVARIABLES
/BREAK = x
/sdY = SD(Y)
/meanY = MEAN(Y).

DATASET DECLARE linreg.
REGRESSION
/MISSING LISTWISE
/STATISTICS COEFF OUTS R ANOVA
/CRITERIA = PIN (.05) POUT (.10)
/NOORIGIN
/DEPENDENT sdY
/METHOD = ENTER meanY
/OUTFILE = COVB (linreg).

* make a plot in main output window for later inspection
OUTPUT ACTIVATE mainOutput.
SET RESULTS = LISTING.
TSET NEWVAR = NONE.
CURVEFIT
/VARIABLES = sdY WITH meanY
/NOCONSTANT
/MODEL = LINEAR
/PLOT FIT.
WLS calibration algorithm

```plaintext
52  OUTPUT ACTIVATE dummyOutput.
53  SET RESULTS = NONE.
54  DATASET ACTIVATE linreg.
55  FLIP VARIABLES=CONST_ meanY
56  /NEWNAMES=ROWTYPE._.
57  DATASET NAME linreg2 WINDOW=FRONT.
58  FORMATS SIG(F8.6) EST(F16.8).
59  SELECT IF (CASE_LBL="meanY").
60  EXECUTE.
61  WRITE OUTFILE="C:\ TEMP\ regressingWeights.sps"
62    /"COMPUTE sdC1 = " EST ".".
63  EXECUTE.
64
65  * prepare estimated weights for regression.
66  DATASET ACTIVATE calibration.
67  DATASET CLOSE linreg.
68  DATASET CLOSE linreg2.
69  INSERT FILE ="C:\ TEMP\ regressingWeights.sps"
70  EXECUTE.
71
72  AGGREGATE
73    /OUTFILE=* MODE=ADDVARIABLES
74    /BREAK=
75    /calMin=MIN(!stdVals)
76    /calMax=MAX(!stdVals).
77  EXECUTE.
78  * conditionally assign weights, true or estimated SD depending on
79  user choice.
80  !IF (!trueSD=1) !THEN
81    COMPUTE w=1/(sdY)**2.
82  EXECUTE.
83  AGGREGATE
84    /OUTFILE=* MODE=ADDVARIABLES
85    /BREAK=
86    /w_min=MIN(w)
87    /sdY_mean=MEAN(sdY).
88  IF (w_min=0) w=1/(sdY/sdY_mean)**2.
89  EXECUTE.
90  DELETE VARIABLES sdY_mean w_min.
91  !IFEND
92
93  !IF (!trueSD=0) !THEN
94    COMPUTE w=1/(sdC1*meanY)**2.
95  EXECUTE.
96  AGGREGATE
97    /OUTFILE=* MODE=ADDVARIABLES
98    /BREAK=
99    /w_min=MIN(w)
```

[214]
WLS calibration algorithm

99  /sdY_mean=MEAN(sdY).
100  IF (w_min=0) w=1/((sdC1*meanY)/sdY_mean)**2.
101  EXECUTE.
102  DELETE VARIABLES sdY_mean w_min.
103  !IFEND
104
105  **************** quadratic model ****************.
106  * section for all of the weights and x multipliers for quadratic.
107  COMPUTE wx = w*x.
108  COMPUTE wx2 = w*x**2.
109  COMPUTE wx3 = w*x**3.
110  COMPUTE wx4 = w*x**4.
111  COMPUTE wxy = w*x*y.
112  COMPUTE wx2y = w*x**2*y.
113  COMPUTE wy = w*y.
114  COMPUTE x2 = x**2.
115  COMPUTE dummy=1.
116  EXECUTE.
117  AGGREGATE
118  /OUTFILE=ADDVARIABLES /BREAK =dummy
119  /Sw = SUM(w)
120  /Swx = SUM(wx)
121  /Swx2 = SUM(wx2)
122  /Swx3 = SUM(wx3)
123  /Swx4 = SUM(wx4)
124  /Swxy = SUM(wxy)
125  /Swx2y = SUM(wx2y)
126  /Swy = SUM(wy)
127  /Sx2 = SUM(wx2)
128  /Nc = NU(!stdVals).
129  COMPUTE xBar = Swx/Sw.
130  COMPUTE x2Bar = Swx2/Sw.
131  COMPUTE yBar = Swy/Sw.
132  COMPUTE sxx = Swx2-Swx*xBar**2.
133  COMPUTE sfx = Swx3 - Sw*xBar*x2Bar.
134  COMPUTE sff = Swx4-Swx2Bar**2.
135  COMPUTE sxy = Swxy-Swx*xBar*yBar.
136  COMPUTE sfy = Swx2y - Sw*x2Bar*yBar.
137  EXECUTE.
138
139  * weighted residuals of the intercept-only model, and their sums.
140  COMPUTE yRsq = w*(y-yBar)**2.
141  EXECUTE.
142  AGGREGATE
143  /OUTFILE=ADDVARIABLES
144  /BREAK =dummy
145  /SyRsq = SUM(yRsq).
* common denominator for Quad WLS.
COMPUTE Del1 = sxx*sff-sfx**2.
EXECUTE.

* quad WLS parameters and estimate curve points.
COMPUTE b1 = (sff*sxy-sfx*sfy)/Del1.
COMPUTE b2 = (sxx*sfy-sfx*sxy)/Del1.
EXECUTE.
COMPUTE yHatQ = yBar + b1*(x-xBar) + b2*(x**2-x2Bar).
EXECUTE.

* weighted quad model residuals and their sums.
COMPUTE RsqQ = w*(y-yHatQ)**2.
EXECUTE.
AGGREGATE
/OUTFILE=* MODE=ADDVARIABLES
/BREAK =dummy
/SRsqQ = SUM(RsqQ).

* residual variance of the model.
COMPUTE sRQ = sqrt(SRsqQ / (Nc-3)).
EXECUTE.

* the Scheffe coefficient and the common radical term for the bands.
COMPUTE Fq=sqrt(3*IDF.F(1-!alpha/2,3,Nc-3)).
COMPUTE U = 1/Sw + (x-xBar)**2*sff/Del + (x**2-x2Bar)**2*sxx/Del
   -2*(x-xBar)*(x**2-x2Bar).
EXECUTE.

************** linear model **************.
* analogous multipliers (simpler than quad).
COMPUTE wDxy = w*(x-xBar)*y.
COMPUTE wDx2 = w*(x-xBar)**2.
EXECUTE.
AGGREGATE
/OUTFILE=* MODE=ADDVARIABLES /BREAK =dummy
/Swdxy = SUM(wDxy)
/SwDx2 = SUM(wDx2).

* lin WLS parameter and estimate curve points.
COMPUTE b0=Swdxy/SwDx2.
EXECUTE.
COMPUTE yHatL = yBar + b0*(x-xBar).
EXECUTE.

* weighted linear model residuals and their sums.
COMPUTE RsqL = w*(y-yHatL)**2.
EXECUTE.
WLS calibration algorithm

AGGREGATE
/OUTFILE=** MODE=ADDVARIABLES
/BREAK=dummy
/SRsqL = SUM(RsqL).
* residual variance of the model.
COMPUTE sRL = sqrt(SRsqL / (Nc-2)).
EXECUTE.

* compare linear and quadratic and convert quadratic to linear
should it be needed.
* if linear is better: .
DO IF ((SRsqL - SRsqQ)/ sRQ **2 < IDF.F(1-!alpha,1,Nc-3)).
  COMPUTE b1 = b0.
  COMPUTE Del = SwDx2.
  COMPUTE b2=0.
  COMPUTE sxx=0.
  COMPUTE sfx=0.
  COMPUTE sff=1.
  COMPUTE Rsq = 1-SRsqL/SyRsq.
  COMPUTE Fs = sqrt(2*IDF.F(1-!alpha/2,2,Nc-2)).
  COMPUTE sR = sRL.
ELSE.
  COMPUTE Rsq = 1-SRsqQ/SyRsq.
  COMPUTE Fs = sqrt(3*IDF.F(1-!alpha/2,3,Nc-3)).
  COMPUTE sR = sRQ.
END IF.
EXECUTE.

* getting the true replicate, mean, and SD for ALL groups.
* needed for t distribution used in calibration.
AGGREGATE
/OUTFILE=** MODE=ADDVARIABLES
/BREAK=Split
/M=nu (!Mxs)
/yAve = mean (!Mxs)
/ySD = SD (!Mxs).
COMPUTE ySE = ySD / sqrt(m).
COMPUTE tInv = Idf.t(1-!alpha/2,m-1).
EXECUTE.

* inverse regression results, depending on what kind of model was
used.
IF (b1<0 AND b2<>0) xInv = -(b1+(4*b2*yAve-4*b2*yBar+4*b2**2*x2Bar+
b1**2+4*b1*b2*xBar)**(1/2))/(2*b2).
IF (b1>0 AND b2<>0) xInv = -(b1-(4*b2*yAve-4*b2*yBar+4*b2**2*x2Bar+
b1**2+4*b1*b2*xBar)**(1/2))/(2*b2).
IF (b2=0) xInv = (yAve - yBar + b1*xBar)/b1.
EXECUTE.

* NLR to iteratively solve for CI max.
SORT CASES BY split.
SPLIT FILE BY split.
SET RESULTS = NONE.
DATASET DECLARE temp.
OMS

/SELECT TABLES
/IF COMMANDS=['Nonlinear Regression Analysis'] SUBTYPES=['
Parameter Estimates']
/DESTINATION FORMAT=SAV NUMBERED=TableNumber_
OUTFILE='temp'.
* Nonlinear Regression.
MODEL PROGRAM max =1.
COMPUTE PRED_ = yBar + b1*max - b2* x2Bar - b1* xBar - tInv * ySE + b2* max **2 -
Fs*sR*(1/Sw+(sff*(max-xBar)**2)/Del+(sxx*(x2Bar-max**2)**2)/Del
+(2*sfx*(x2Bar-max**2)*(max-xBar))/Del)**(1/2).
NLR yAve
/OUTFILE='C:\Users\Reece\AppData\Local\Temp\SPSSFNLR.TMP'
/PRED PRED_
/Criteria SCONVERGENCE 1E-8 PCON 1E-8.
OMSEND.
DATASET ACTIVATE temp.
WRITE OUTFILE='C:\temp\maxval.sps'
/'IF ( split=' Var1 ') ' Var2 '=' Estimate ' , '/'
EXECUTE.'.
EXECUTE.
DATASET ACTIVATE calibration.
INSERT FILE = 'C:\temp\maxval.sps'.
RENAME VARIABLES ( max=xMaxInv).
DATASET CLOSE temp.

* do it again for the min.
DATASET DECLARE temp.
OMS

/SELECT TABLES
/IF COMMANDS=['Nonlinear Regression Analysis'] SUBTYPES=['
Parameter Estimates']
/DESTINATION FORMAT=SAV NUMBERED=TableNumber_
OUTFILE='temp'.
* Nonlinear Regression.
MODEL PROGRAM min =1.
COMPUTE PRED_ = yBar + b1*min - b2* x2Bar - b1* xBar + tInv * ySE + b2* min **2 -
Fs*sR*(1/Sw+(sff*(min-xBar)**2)/Del+(sxx*(x2Bar-min**2)**2)/Del
+(2*sfx*(x2Bar-min**2)*(min-xBar))/Del)**(1/2).
NLR yAve
/OUTFILE='C:\Users\Reece\AppData\Local\Temp\SPSSFNLR.TMP'
\textbf{WLS calibration algorithm}

\begin{verbatim}
281       /PRED PRED_
282       /CRITERIA SSCONVERGENCE 1E-14 PCON 0.
283       OMS END.
284       DATASET ACTIVATE temp.
285       WRITE OUTFILE='C:\temp\minval.sps'
286         /'IF ( split=' Var1 ') ' Var2 ' = ' Estimate '. '/'
287         EXECUTE.'.
288       EXECUTE.
289       DATASET ACTIVATE calibration.
290       INSERT FILE='C:\temp\minval.sps' .
291       RENAME VARIABLES (min=xMinInv).
292       DATASET CLOSE temp.
293       split file off .
294         * critical value of dependent var based on alpha .
295         COMPUTE lC = yBar -b1*xBar -b2*x2Bar +Idf.t(1-!alpha,Nc-(2+(b2<>0)))*
296           sR*sqt((sdC1*(yBar-b1*xBar-b2*x2Bar))**2/!bandsM+1/Sw +(xBar)
297           **2*sff/Del + (x2Bar)**2*sxx/Del-2*(-xBar)*(-x2Bar)*sfx/Del).
298         EXECUTE.
299         * regress the limit of detection based on beta .
300         COMPUTE TB = IDF.t(!beta,Nc-(2+(b2<>0))).
301         EXECUTE.
302         SET RESULTS = NONE .
303         DATASET DECLARE temp .
304           /SELECT TABLES
305             /IF COMMANDS=["Nonlinear Regression Analysis"]
306             SUBTYPES=['
307               Parameter Estimates ']
308             /DESTINATION FORMAT=SAV NUMBERED=TableNumber_
309             OUTFILE='temp'.
310         * NonLinear Regression .
311         MODEL PROGRAM xD=1.
312         COMPUTE PRED_ = yBar -b1*(xBar-xD)-b2*(x2Bar-xD**2)+
313           sR*tB*(1/Sw+(sdC1*(yBar-b1*(xBar-x))-b2*(x2Bar-x**2)))**2/!bandsM+
314           (sff*(xBar-xD)**2)/Del+(sxx*(x2Bar-xD**2)**2)/Del-(2*sfx*(
315             x2Bar-xD**2)*((xBar-xD))/Del)**(1/2).
316         NLR 1C
317           /OUTFILE='C:\Users\Reece\AppData\Local\Temp\SPSSFNLR.TMP'
318       /PRED PRED_.
319       /CRITERIA SSCONVERGENCE 1E-8 PCON 1E-8.
320       OMS END.
321       DATASET ACTIVATE temp .
322       WRITE OUTFILE='C:\temp\xD.sps'
323         /' COMPUTE xD = ' Estimate '. '/' EXECUTE.'.
324       EXECUTE.
325       DATASET ACTIVATE calibration.
326       INSERT FILE='C:\temp\xD.sps' .
\end{verbatim}
DATASET CLOSE temp.

* compute confidence and prediction bands.
COMPUTE tC = IDF.t(1-!alpha/2,Nc-(2+(b2<>0))).
EXECUTE.

* summary of parameters.
data list list (",")
   /dummy (F8.0) xXLS (F8.0) YXLS (A750) yMax (A750) yMin (A750)
   yBandMax (A750) yBandMin (A750).
BEGIN DATA.
1, 0, " " , " " , " " , " " , " " , " "
END DATA.
DATASET NAME excelOutput WINDOW=FRONT.
DATASET ACTIVATE calibration.
COMPUTE dummy = $CASENUM.
EXECUTE.
SORT CASES BY dummy.
DATASET ACTIVATE excelOutput.
MATCH FILES FILE=* TABLE=calibration BY dummy.
EXECUTE.
COMPUTE tC = IDF.t(1-!alpha/2,Nc-(2+(b2<>0))).
EXECUTE.
OUTPUT ACTIVATE mainOutput.
FORMATS Rsq (F16.12) yBar (E15.5) b1(E15.5) xBar(E15.5) b2(E15.5)
   x2Bar(E15.5) tC(E15.5) sR(E15.5) sW(E15.5) sff(E15.5) Del(E15.5)
   sxx(E15.5) sfx (E15.5).
SUMMARIZE
   /TABLES= Rsq yBar b1 xBar b2 x2Bar tC sR sW sff Del sxx sfx
   /CELLS = NONE
   /FORMAT= NOCASENUM list nototal nocasenum
   /MISSING=VARIABLE.
SUMMARIZE
   /TABLES= Rsq
   /CELLS = NONE
   /FORMAT= NOCASENUM list nototal nocasenum
   /MISSING=VARIABLE.
SET RESULTS = NONE.
OUTPUT ACTIVATE dummyOutput.
DATASET ACTIVATE calibration.
DATASET CLOSE excelOutput.

* dataset with regression values summarized.
dataset copy summary.
DATASET ACTIVATE summary.
WLS calibration algorithm

```plaintext
368  SORT CASES BY !grpVar.
369  MATCH FILES file=*  
370     /BY !grpVar
371     /first=first.
372  EXECUTE.
373  SELECT IF (first).
374  EXECUTE.
375  DELETE VARIABLES first.
376
377  * construct curve dataset so cal curve can be graphed.
378  NEW FILE.
379  INPUT PROGRAM.
380     LOOP x=0 TO !curveSegs.
381        COMPUTE dummy=1.
382     END CASE.
383  END LOOP.
384  END FILE.
385  END INPUT PROGRAM.
386  EXECUTE.
387  DATASET NAME curves.
388  DATASET ACTIVATE curves.
389  MATCH FILES / FILE =*
390     /TABLE = summary
391     /BY= dummy
392     /KEEP lC, tC, xD, xBar x2Bar yBar, b1, b2, sdC1, calMin, calMax,
393                  Del, sfx, sxx, sff, sW, sR.
394  EXECUTE.
395  COMPUTE x= calMin+( $casenum -1)/! curveSegs*( calMax - calMin).
396  EXECUTE.
397  COMPUTE yPred = yBar -b1*( xBar-x)-b2*(x2Bar-x**2).
398  EXECUTE.
399  COMPUTE yMax = yBar-b1*(xBar-x)-b2*(x2Bar-x**2)+sR*tC*(1/Sw+(sdC1*
400             yPred)**2/!bandsM+(sff*(xBar-x)**2)/Del+(sxx*(x2Bar-x**2)**2)/
401             Del-(2*sfx*(x2Bar-x**2)*(xBar-x))/Del)**(1/2).
402  COMPUTE yMin = yBar-b1*(xBar-x)-b2*(x2Bar-x**2)-sR*tC*(1/Sw+(sdC1*
403             yPred)**2/!bandsM+(sff*(xBar-x)**2)/Del+(sxx*(x2Bar-x**2)**2)/
404             Del-(2*sfx*(x2Bar-x**2)*(xBar-x))/Del)**(1/2).
405  COMPUTE yBandMax = yBar-b1*(xBar-x)-b2*(x2Bar-x**2)+sR*tC*(1/Sw+(sff*
406             (xBar-x)**2)/Del+(sxx*(x2Bar-x**2)**2)/Del-(2*sfx*(x2Bar-x*
407             **2)*(xBar-x))/Del)**(1/2).
408  COMPUTE yBandMin = yBar-b1*(xBar-x)-b2*(x2Bar-x**2)-sR*tC*(1/Sw+(sff*
409             (xBar-x)**2)/Del+(sxx*(x2Bar-x**2)**2)/Del-(2*sfx*(x2Bar-x*
410             **2)*(xBar-x))/Del)**(1/2).
411  EXECUTE.
412  FORMATS x(F8.5) yPred(F8.5) yMax(F8.5) yMin(F8.5) yBandMax(F8.5)
413                  yBandMin(F8.5).
414  EXECUTE.
415
```

[221]
Because most of the code can be called remotely, use of the WLS algorithm requires little additional code. Lines 1 and 14 of code block 7.2 are intended to keep the SPSS window from becoming too cluttered with intermediate calculations. Line 2 is optional so long as the entirety of code block 7.1 is run once in the SPSS session before code block 7.2 is run. For a description of the key values, see Table 7.3. Code block 7.2 is completed to analyze the example dataset in Table 7.2.
7.3 Assay reproducibility metrics

The reproducibility of the MPO activity on a polymer surface (MAPS) assay was determined taking into consideration the uncertainty in each individual assay, resulting in more robust parameters than the traditional standard deviation and coefficient of variation. These parameters were calculated by adapting methods designed to combine variances from independent experiments for meta-analyses. Similar to Section §7.2, Table 7.4 provides a list of parameter definitions used for these calculations. The method is presented symbolically...
Assay reproducibility metrics

to describe the general case; parameter values used in evaluating the MAPS assay are noted where applicable.

Each patient sample $q$ was analyzed with a total of $T$ independent replications; each replicated assay was performed with internal technical replicates $m$. For each of the independent replicates $r$, the variance $s_{qr}^2$ was back-calculated from the $1 - \alpha$ confidence interval about the predicted mean. The variance was then used to weight each assay result:

$$
\hat{w}_{qr} = \frac{1}{s_{qr}^2} \left( \frac{\sqrt{m_{qr}} (\bar{x}_{qr,\text{max}} - \bar{x}_{qr,\text{min}})}{2 t_{1-\alpha/2,m-1}} \right)^{-2} \quad (7.40)
$$

Next, the weighted mean is calculated for each sample:

$$
\hat{x}_q = \frac{\sum_{r=1}^{T} \hat{w}_{qr} \bar{x}_{qr}}{\sum_{r=1}^{T} \hat{w}_{qr}} \quad (7.41)
$$

An estimate of the heterogeneity of variance $\hat{\Delta}^2$ is calculated, which adds additional variance to the final reproducibility statistic based on how widely dispersed the estimates and variances are for each sample.

$$
\hat{\Delta}_q^2 = \max \left( 0, \frac{\sum_{r=1}^{T} \hat{w}_{qr} (\bar{x}_{qr} - \bar{x}_q)^2 - (T - 1)}{\sum_{r=1}^{T} \hat{w}_{qr} - \left( \sum_{r=1}^{T} \hat{w}_{qr}^2 \right) \left( \sum_{r=1}^{T} \hat{w}_{qr} \right)^{-1}} \right) \quad (7.42)
$$

New weights for each replicate are then computed accounting for $\hat{\Delta}_q^2$:

$$
\hat{w}_{qr} = \frac{1}{\hat{w}_{qr} + \hat{\Delta}_q^2} \quad (7.43)
$$

This had the effect of increasing the relative weight given to uncertain measurements if the spread among replications was wide. From these corrected weights, a new weighted mean

---

7With regards to Chapter 4, the MAPS assay was replicated four times with technical triplicates, $[T, m] = [4, 3]$

8$\alpha = 0.05$ from the 95% confidence intervals

9Although the confidence intervals were not technically symmetric about the mean, the deviation from symmetry was not great enough to create a noticeable impact on the final reproducibility metrics.

[224]
were calculated as previously along with a weighted standard deviation from the standard error of the weighted mean:

\[
\tilde{x}_q = \frac{\sum_{r=1}^{T} \tilde{w}_{qr} \tilde{x}_{qr}}{\sum_{r=1}^{T} \tilde{w}_{qr}}
\]

\[
\tilde{s}_q = \sqrt{\frac{T}{\sum_{r=1}^{T} \tilde{w}_{qr}}} \tilde{\Delta}_q
\]

Lastly, the coefficient of variability is computed in the same manner as a more traditional coefficient of variation:

\[
\omega = \frac{\tilde{s}_q}{\tilde{x}_q}
\]
7.4 ImageJ macros

7.4.1 Quadrangular ROI array

This ImageJ macro was written to define an array of identical elliptical regions of interest (ROIs) to ensure that quantification of luminescence from microtiter plate wells would not be confounded by varying ROI position or size. It was based on a previous text-editing script written to aid other lab members with grid ROIs made in IVIS image analysis software Living Image. The macro version was written shortly after the arrival of the ONYX/M imaging system, the data from which are best analyzed in the open-source ImageJ. The macro can be installed to ImageJ or any of its builds, e.g., Fiji, containing the ROI manager tool. It can also be run from the text dialog. Instructions can be found in the macro comment header.

Source Code 7.3: Quadrangular ROI array

```
1 // This plugin makes quadrangular arrays of ROIs defined by 4 corners;
2 // The ROIs are labeled with grid, row, and column numbers and stored in the ROI manager;
3 // This is intended for analyzing microtiter plate images, originally developed for bioluminescence;
4 // written by Reece Goiffon, rjgoiffon@wustl.edu;
5 // Washington University School of Medicine, 2010-2013;
6 // last tested and updated with the Fiji distribution of ImageJ 1.47q;
7
8 // Instructions for a grid of I rows and J columns:
9 // 1. draw and place an elliptical selection at Row1,Column1;
10 // 2. add this ROI to the ROI manager (default shortcut: t);
11 // 3. drag the selection to Row1,ColumnJ and add to the ROI manager;
12 // 4. drag the selection to RowI,Column1 and add to the ROI manager;
13 // 5. drag the selection to RowI,ColumnJ and add to the ROI manager;
14 // 6. (optional) define more grids by continuing to drag the selection and adding to the ROI manager;
15 // total number of ROIs must be N*4 where N is the number of arrays desired;
16 // 7. run the macro and enter the desired number of rows/columns per array. all arrays have the same I and J;
17```

18 macro "Quadrangular ROI array" {
19   Nrows=8;
20   Ncols=12;
21   Ngrids = 1;
22
23   Dialog.create("Grid ROI creator");
24   Dialog.addMessage("You must have placed 4 corner ROIs into the ROI
               manager for each grid \n Make sure that 1 is diagonal from 4
               \n Note that rotation doesn’t matter.");
25   Dialog.addNumber("Rows:", Nrows);
26   Dialog.addNumber("Columns:", Ncols);
27   Dialog.show();
28   Nrows = Dialog.getNumber();
29   Ncols = Dialog.getNumber();
30
31   R = roiManager("count");
32   getDimensions(W, H, c, s, f);
33   if (floor(R/4)!=(R/4)) {
34       exit("You need four corner ROIs per grid.");
35   }
36   setBatchMode(true);
37   Ngrids = R / 4;
38   ROIs=Nrows*Ncols*Ngrids;
39
40   cornerX = newArray(R);
41   cornerY = newArray(R);
42   for (i=0; i<4*Ngrids; i++) {
43       roiManager("select", i);
44       getSelectionBounds(x, y, w, h);
45       cornerX[i] = x;
46       cornerY[i] = y;
47   }
48
49   Xs = newArray(Ngrids*Nrows*Ncols);
50   Ys = newArray(Ngrids*Nrows*Ncols);
51   for (g=0; g<Ngrids; g++) {
52       for (i=0; i<Nrows; i++) {
53           for (j=0; j<Ncols; j++) {
54               jj=j/(Ncols-1);
55               ii=i/(Nrows-1);
56
57               Xs[g*Nrows*Ncols+i*Ncols+j] = cornerX[g*4]*(1-jj)*(1-ii) +cornerX[g*4+1]*(jj)*(1-ii) + cornerX[g*4+2]*(1-jj)*(ii) + cornerX[g*4+3]*(ii)*(jj);
58
59               Ys[g*Nrows*Ncols+i*Ncols+j] = cornerY[g*4]*(1-jj)*(1-ii) +cornerY[g*4+1]*(jj)*(1-ii)
60           }
61       }
62   }
63

roiManager("Reset");
newImage("temp", "32-bit Black", W, H, 1);
names=newArray(ROIs);
for (g=0; g<Ngrids; g++) {
    for (i=0; i<Nrows; i++) {
        for (j=0; j<Ncols; j++) {
            k = g*Ncols*Nrows + i*Ncols + j;
            selectWindow("temp");
            run("Specify...", "width="+w+" height="+h+" x="+Xs[k]+" y="+Ys[k]+" oval");
            roiManager("Add");

            if(j<9) {
                colName = "00"+(j+1);
            } else if (j<99) {
                colName = "0"+(j+1);
            } else {
                colName = j+1;
            }

            if(i<9) {
                rowName = "00"+(i+1);
            } else if (i<99) {
                rowName = "0"+(i+1);
            } else {
                rowName = i+1;
            }

            if(g<9) {
                gridName = "00"+(g+1);
            } else if (g<99) {
                gridName = "0"+(g+1);
            } else {
                gridName = g+1;
            }

            roiManager("Select", k);
            roiManager("Rename","G"+gridName+"R"+rowName+"C"+colName);
        }
    }
}
selectWindow("temp");
close();
Vignetting is the non-uniform intensity of an imaging system due to the optical properties of camera apertures. Proprietary image analysis software for use with out-of-the-box imaging platforms such as the IVIS100 correct for vignetting\textsuperscript{10}, but the imaging conditions in the ONYX/M are too customizable to offer universal correction settings. By using techniques from the optical engineering literature, the ONYX/M could be calibrated for any stage height, iris aperture, and focal length using a single test image, non-linear regression, and the following ImageJ macro.\textsuperscript{8,9}

The camera was first focused on a grid test pattern, which was replaced with a clean, unbent sheet of 20 lb. copy paper. A 1 min mean intensity exposure was taken with the ONYX/M illumination set to result in the intensity distribution near the middle of pixel saturation. (Figure 7.1B) Dead pixels were removed to prevent bias and the image was fit to the equation:

\begin{equation}
S = C \cos^4 \theta = C \cos^4 \left( \tan^{-1} \left( \frac{r}{A} \right) \right)
\end{equation}

where \( r \) was calculated as the radial distance / pixels from the image center, \( S \) is signal intensity, and \( C = S | r = 0 \). (Figure 7.1A) From these regression parameters, a noiseless image was calculated and normalized such that peak intensity \( C = 1 \) resulting in the calibration matrix \( C \). (Figure 7.1C) When run on an image stack, the ImageJ macro computes the

\textsuperscript{10}often called “flat-field correction”
Figure 7.1: ONYX imaging vignette correction. (A) The principle of the vignette correction. An image of a white, matte surface with even illumination at the desired focal plane is fit to equation (7.47), the standard reflectance map equation.8,9 (B) Example calibration image. Noise is the sum of camera noise (both random and systematic due to sensor defects) and an interference pattern from the array of lights illuminating the stage. (C) Calibration matrix computed from regression parameters obtained from the image in (B) and equation (7.47). (D) Back-corrected version of the image shown in (B) processed with equation (7.48) and correction matrix shown in (C).

Hadamard quotient of each stack image matrix $I$ and the calibration image matrix:

$$I_{\text{corrected}} = I \odot C$$  \hspace{1cm} (7.48)

Some deviation from model equation (7.48) due to the non-uniform illumination from the ONYX stage lights results in some non-uniformity when the transformation is applied to the original calibration image. (Figure 7.1D) Despite this slight imperfection, systematic variance in experimental imaging sequences were greatly reduced with this technique.
Source Code 7.4: ONYX imaging vignette correction

```c
// This macro is required to fix the images obtained on the Onyx, which have systematic bias due to vignetting;
// A calibration image must be taken with even, moderate illumination with the focus and stage settings identical to the images to be corrected;
// this calibration image must then be fit to C*(cos(arctan(r/A)))^4;
// where r is the radius (pixels) from image center and C, A require fitting;
// from the regression parameters, calculate a calibration image from r and the obtained value of A (set C=1);
// this image path needs to be added to lines 10 and 11, the filename to line 14;

macro "ONYX vignette correction"{
    process = getTitle();
    open("<insert path of calibration image>");
    imageCalculator("Divide create 32-bit stack",process,"<insert path of calibration image>");
    selectWindow("Result of "+process);
    rename("CORRECTED "+process);
    selectWindow("<insert name of calibration image>");
    close()
    selectWindow("CORRECTED "+process);
}
```

7.5 References


