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Proteolytic Activity Matrix Analysis (PrAMA) for Simultaneous Determination of Multiple Protease Activities

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Abstract

Matrix metalloproteinases (MMPs) and A Disintegrin and Metalloproteinases (ADAMs) are two related protease families that play key roles in matrix remodeling and growth factor ligand shedding. Directly ascertaining the proteolytic activities of particular MMPs and ADAMs in physiological environments in a non-invasive, real-time, multiplex manner remains a challenge. This work describes Proteolytic Activity Matrix Analysis (PrAMA), an integrated experimental measurement and mathematical analysis framework for simultaneously determining the activities of particular enzymes in complex mixtures of MMPs and ADAMs. The PrAMA method interprets dynamic signals from panels of moderately specific FRET-based polypeptide protease substrates to deduce a profile of specific MMP and ADAM proteolytic activities. Deconvolution of signals from complex mixtures of proteases is accomplished using prior data on individual MMP/ADAM cleavage signatures for the substrate panel measured with purified enzymes. We first validate PrAMA inference using a compendium of roughly 4000 measurements involving known mixtures of purified enzymes and substrates, and then demonstrate application to the live-cell response of wildtype, ADAM10−/−, and ADAM17−/− fibroblasts to phorbol ester stimulation. Results indicate PrAMA can distinguish closely related enzymes from each other with high accuracy, even in the presence of unknown background proteolytic activity. PrAMA offers a valuable tool for applications ranging from live-cell in vitro assays to high-throughput inhibitor screening with complex enzyme mixtures. Moreover, our approach may extend to other families of proteases, such as caspases and cathepsins, that also can lack highly-specific substrates.

Keywords

Metalloproteinase activity; analytical biochemistry; computational enzyme kinetics

Introduction

Matrix metalloproteinases (MMPs) comprise a family of 23 zinc-dependent endopeptidases that are part of the Metzincin family of enzymes and are generally active on or near the cell surface [1, 2]. As central regulators of extracellular microenvironments throughout the human body, MMPs play key roles in normal physiological processes including development [3], angiogenesis [4, 5], tissue remodeling [6], wound repair, and inflammation [7]. On the other hand, they are also implicated in a wide array of pathologies, ranging from cancer, tumor invasion, and metastasis [8], to respiratory diseases such as asthma and

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chronic obstructive pulmonary disease (COPD) [9]. MMP proteolytic activities are tightly controlled. Once active, certain MMPs (e.g., MMP2) have been demonstrated to act on hundreds of endogenous substrates [10, 11]. MMP substrates include signaling molecules (e.g., cytokines, chemokines, growth factors, GPCRs, growth factor receptors, and cytokine receptors), extracellular matrix components (e.g., collagen, laminin, and fibronectin), cell adhesion molecules, clotting and complement cascade proteins, and proteases themselves [12–15].

Closely related to MMPs, ADAM (A Disintegrin and Metalloproteinase) enzymes are metalloproteinases (MPs) within the Metzincin family that are mostly bound at the cell surface [16]. At least 13 ADAMs existing in humans have intact metalloproteinase domains and proteolytic activity [14]. ADAMs mediate various cellular behaviors including migration, adhesion [17], proliferation [15], and apoptosis [16, 18]. Similar to MMPs, ADAM family enzymes are found throughout the body and support diverse physiological processes such as development [15] and angiogenesis [5, 16]. Likewise, ADAMs can become dysregulated in a variety of diseases and play roles in pathologies including cancer [1, 18], inflammatory bowel disease, and asthma [16]. Current research suggests that ADAMs have a narrower repertoire of substrates compared to MMPs, and principally function to shed the ectodomain of surface-bound proteins such as growth factor ligands, growth factor receptors, cell adhesion molecules, and cytokine receptors [14, 15].

Three key properties of MP biology have created the need for methods that directly observe protease activity in a specific, non-invasive, real-time, and multiplex manner. First, the extensive amount of post-translational modification and regulatory mechanisms controlling MP proteolytic activity make direct activity measurements a valuable and complementary addition to common methods of assessing protein function, such as western blotting, immunohistochemistry, and genetic manipulation [19–22]. Second, the plethora of endogenous substrates cleaved by certain MPs, the context dependency of endogenous substrate cleavage, and the overlapping endogenous substrate specificity of closely related MPs make it difficult to quantitatively match the contributions of specific proteases to global observations of endogenous substrate degradation [11, 19, 23]. Quantitative determination of selected protease activities would complement measurements that focus on endogenous substrate cleavage, thereby facilitating attempts to match particular proteolytic activities to patterns of substrate degradation. Third, cyclical feedback interactions and compensatory mechanisms among closely related MPs can severely complicate the interpretation of protease network interactions [15, 24, 25]. Non-invasive and multiplexed measurement of MP activity would allow for the assessment of protease network interactions without artificially biasing the underlying network structure.

While many useful methodologies currently exist to study MPs, unfortunately none simultaneously allow for direct, non-invasive, multiplex, real-time measurements of specific protease activity. In general, existing methods such as zymography, activity based probes, and mass-spectrometry based methods all must choose between invasiveness, specificity, and throughput [10, 26–30]. Synthetic polypeptide protease substrates have been extensively developed to directly assess MP activity in a non-invasive and real-time manner [20, 31, 32]. These substrates typically consist of a fluorescence resonance energy transfer (FRET) donor and quencher fluorophore that are separated by a 3–10 amino acid linker containing a protease cleavage motif. Upon cleavage of the polypeptide linker, the donor fluorophore separates from the quencher and fluorescence increases. Protease activity dynamics can then be tracked by observing the change in fluorescence over time. Similarly to many endogenous MP substrates, MP FRET-substrates are generally cleaved by multiple closely related proteases [32–34]. Several strategies, including positional scanning of synthetic combinatorial libraries [33, 35] and directed evolution using phage display [36] have
attempted to optimize substrate sequences such that they are more selectively cleaved by a specific protease. Combinations of multiple substrates and inhibitors have also been implemented to increase specificity [37]. These strategies often succeed at distinguishing between two or a few proteases, but cross-reactivity nevertheless remains problematic in more complex mixtures [33, 38, 39].

This work describes an approach we term ‘Proteolytic Activity Matrix Analysis’ (PrAMA) as a method of using panels of FRET-substrates to infer a dynamic, quantitative, and specific profile of MMP and ADAM proteolytic activities. PrAMA ascertains specific protease activity by deconvoluting from measurements derived from relatively non-specific FRET-substrates, employing prior knowledge of individual MMP/ADAM cleavage signatures ascertained using purified enzymes. This approach allows PrAMA to elucidate particular enzyme activities from cleavage signatures obtained in complex samples containing multiple proteases. The integrated experimental measurement and mathematical analysis framework exploits the advantages of FRET-substrates, which support non-invasive real-time measurements of live-cell activity, while addressing their problems of limited specificity and multiplexing. Peptide library microarrays have been previously implemented to assess global patterns of protease activity and infer specific protease activity [40]. Nevertheless, PrAMA’s novel combination of mathematical and experimental methodologies allows for greater quantification of protease activity, lower expense, and higher throughput compared to microarray-based approaches. Ultimately, PrAMA fills a niche that complements many other current methods of assaying MP activity and substrate degradation. This niche is especially important for multivariate network-level analysis, where the ability to simultaneously measure multiple MP activities in a non-invasive, real-time, and relatively high-throughput manner confers the greatest benefits. We anticipate that such network-level approaches will be valuable for designing clinical trials focused on MMPs and for illuminating unintended consequences of the many trials that have failed in the last decade [11, 41].

We present a compendium on the order of 4000 measurements involving mixtures of FRET-substrates and purified recombinant MPs, and use these measurements to both construct the PrAMA inference parameters and test the limits of PrAMA inference accuracy. A priori determination of the PrAMA inference parameters can predict optimal subsets of substrates for distinguishing particular MPs from each other. We demonstrate PrAMA as capable of accurately inferring MP activity even in the presence of background protease activities. Finally, we apply PrAMA to assess the live-cell proteolytic response of wildtype, ADAM10$^{-/-}$, and ADAM17$^{-/-}$ mouse embryonic fibroblasts (MEFs) to phorbol ester stimulation. Overall, this work presents the foundation, validation, and theoretical analysis of a general methodology that has potential applications ranging from systems biology to in vitro inhibitor screening.

Materials and Methods

Materials

Recombinant human ADAMs 8, 9, 10, and 17 were purchased from R & D systems. The catalytic domains of the following recombinant human enzymes were purchased from Enzo Life Sciences: ADAM12 and MMPs 1, 2, 3, 7, 8, 9, 10, 12, 13, and 14. MMP9 Inhibitor I (Cat. No. 444278) was purchased from Calbiochem. GM6001 was obtained from Enzo Life Sciences. Recombinant human TNFα and EGF were obtained from Millipore (Billerica, MA). 18 FRET-substrates were obtained from BioZyme, Inc. Most substrate sequences are currently proprietary. In this work, we refer to substrates as numbers 1–18, and these reference numbers correspond to the following catalog numbers and polypeptide sequences if available: (1) PEPDAB011, (2) PEPDAB012, (3) PEPDAB021, (4) PEPDAB015, (5)
Substrate assays with purified enzymes

For all experiments, substrates were diluted from 5mM stock in dimethyl sulfoxide (DMSO) to a final concentration of 10μM in the appropriate assay buffer. We conducted experiments in four different assay buffers. “ADAM buffer” consists of 20mM Tris, pH 8.0, and 6 × 10^{-4}% Brij-35. “ADAM buffer” also includes 10mM CaCl_2 for experiments involving ADAM8. “MMP buffer” consists of 50mM Tris, pH 7.5, 150mM NaCl, 2mM CaCl_2, 5μM ZnSO_4, and 0.01% Brij-35. We obtained the third buffer, Clonetics™ Mammary Epithelial Cell Basal Media (“MEBM”), as a phenol-red and serum free solution from Lonza, pH 7.4. Final active concentration of MMPs and ADAMs in activity assays ranged from 0.01nM to 7.5nM.

As a fourth buffer, we spiked purified MMP into the conditioned media from the MDA-MB-231 cell line, which is an estrogen receptor negative (ER−) breast cancer cell line derived from the pleural effusion of a breast cancer patient. We obtained these cells from the American Type Culture Collection (ATCC, Manassas, VA) and routinely cultured them at 37°C, 5% CO_2, in DMEM supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100μg/ml streptomycin, 4mM L-Glutamine, and 4.5g/L D-glucose. We collected cell supernatant under the following conditions: cells were grown to 80% confluency in 10cm tissue-culture treated polystyrene plates obtained from Corning Life Sciences (Lowell, MA), serum-starved for 4hrs in basal media consisting of DMEM supplemented with penicillin/streptomycin, and stimulated with basal media supplemented with either 10ng/ml TNFα or 10ng/ml epidermal growth factor (EGF). Supernatant was collected at 12hrs, spun down at 200g for 5mins to remove debris, and immediately flash-frozen. For FRET-substrate assays involving this supernatant, final reactions were composed of a 2:1:1 mixture of 20uM substrate diluted from 5mM DMSO stock into phosphate buffered saline, 4nM active MMP7 diluted in “MMP buffer,” and thawed supernatant.

We determined active site concentrations by comparing observed cleavage rates to previously published catalytic efficiencies for the same substrates in either “MMP Buffer” or “ADAM buffer” [32, 42]. In some cases we performed active site titration with GM6001 to either confirm this comparison or to substitute it when comparison was unavailable. Activity data for active site titrations were fit to the Morrison equation using non-linear least squares curve-fitting (see below). We normalized substrate concentration to a positive control, comprised of 10μM substrate incubated with 0.5% trypsin and 0.2% EDTA (Sigma). Almost all experiments were performed in technical triplicate, except for the MMP7 dilution series and the experiments involving cell supernatant, which both were performed in technical duplicate. For the experiments in triplicate, we excluded clear outliers in a few cases (<10% of all triplicates) using Dixon’s Q-test with a 90% threshold. We performed all experiments at 37°C. In general, readings measured fluorescence approximately every half-hour for roughly five hours. All experimental data are provided in the online supplementary material. We conducted all computational work using Matlab (2009a, MathWorks, Natick, MA).
Live-cell substrate assays

Immortalized wildtype, ADAM17−/−, and ADAM10−/− mouse embryonic fibroblast cell lines were the gift of Carl Blobel and are described elsewhere [19, 25, 43, 44]. Cells were maintained at 37°C, 5% CO₂, in DMEM supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100μg/ml streptomycin, 4mM L-Glutamine, and 4.5g/L D-glucose. For PrAMA, we plate 5000 cells per well in clear-bottom 384-well polystyrene plates from Thermo Scientific (roughly 70,000 cells/cm²²). The following day, we change media and add one of seven FRET substrates (substrates 1, 2, 5, 6, 7, 9, and 15) at 10μM to each well, along with either 1μM phorbol 12-myristate 13-acetate (PMA) or a DMSO control. In all cases media contained <1% DMSO. Following addition of substrate, cells were imaged at 20–40min intervals and at 37°C for 2hrs. We performed experiments in biological quadruplicate and excluded data lying more than 1.5 standard deviations from the mean (at most one sample per quadruplicate).

Enzyme kinetics modeling

We model enzyme kinetics as an extension of the classical Michaelis-Menten (M-M) model, where the initial rate of cleavage, \( V_0 \), is defined as the following: \( V_0 = C_{i,j}[S_i]_0[E_j] \). \( C_{i,j} \) describes the catalytic efficiency, \( k_{cat}/K_m \), with which the \( j \)th enzyme \([E_j]\) cleaves the \( i \)th substrate \([S_i]\). This model assumes \( K_m \gg [S] \), which has been experimentally confirmed for several substrates [45]. In all experiments we aim to infer either the initial rate of substrate cleavage (\( V_0 \)) or the catalytic efficiency (\( C_{i,j} \)) in a reaction, depending on whether \([E]\) is known or unknown (i.e., “self-blinded” in this work), respectively. We infer these parameters by fitting a kinetic model to the time-lapse fluorimetry data, where fluorescence \( F_P(t) \) indicates product formation. Typically, inference using M-M kinetics involves fitting early time-points to the linear M-M model \( F_P(t) = (V_0t + B)F_0/[S]_0 \), where \( B \) is the background signal and \( F_0 \) is the peak fluorescence from the positive control (described above). We extend the linear M-M description to a non-linear “depletion-decay” model. We let \([S]/[S]_0 = 1 - F_P/F_0 \), and describe the observed fluorescence, \( F_{obs} \), in the following equations:

\[
dF_{obs}/dt = V_0(F_0 - F_P)/[S]_0 - k_dF_{obs}
\]  
\[
dF_P/dt = V_0(F_0 - F_P)/(S)_0
\]  

Where \( k_d \) indicates the first-order photobleaching decay of the fluorescent cleavage product. We define a lag-time, \( T_0 \), that denotes the amount of time between the reaction start and the first fluorimetry measurements; \( k_d = 0 \) for \( t < T_0 \). From these equations, \( F_{obs} \) takes the following form:

\[
F_{obs}(t) = F_0 V_0 (e^{-V_0/[S]_0}) (k_d[S]_0 - V_0)^{-1} + Ae^{-k_d}
\]

\[
A = e^{k_d T_0} F_0 ((1 - e^{-V_0/[S]_0}) - V_0/(e^{-V_0/[S]_0}))(k_d[S]_0 - V_0)^{-1}
\]

We fit model parameters in several steps. First, we subtract the signal of a negative control (FRET-substrate only) from all other signals. The maximum fluorescence in the positive control, which is generally at the first time point, indicates \( F_0 \). We determine \( k_d \) from the negative slope of the log-transformed positive control (\( k_d = -\frac{\partial}{\partial t} \ln(F_{pos.cont.}) \)). We obtain the
remaining two parameters ($V_0$ and $T_0$) by non-linear curve-fitting. In several cases, we explicitly measured $T_0$ and compared model fitting with and without explicitly defining that parameter. Results indicate that $V_0$ inference remains consistent regardless of whether $T_0$ is inferred or measured. In cases where $[E]$ is known (i.e. not blinded), we calculate $C_{i,j}$ by the following relation:

$$C_{i,j} = V_0/([S]_0[E_j]).$$

**PrAMA Inference**

PrAMA inference uses panels of FRET-substrate cleavage measurements, coupled with known catalytic efficiencies, $C_{i,j}$, for all relevant $i$ substrates and $j$ enzymes, to infer specific protease activity from a complex mixture of unknown enzymes. PrAMA operates under the assumption that total observed cleavage for the $i$th substrate $V_{0,i}$ in a mixture of enzymes is the summation of cleavages from each individual protease in that mixture:

$$V_{0,i} = [S]_0 \sum C_{i,j} [E_j]$$

(5)

In this application initial substrate concentration is equal among all experiments, allowing it to be simplified as a scalar constant. The complete set of catalytic efficiencies $C_{i,j}$ comprises the model’s $m \times n$ Jacobian matrix for $m$ total substrates and $n$ total enzymes, divided by the substrate concentration:

$$C = \begin{bmatrix}
\frac{\partial V_{0,1}}{\partial [E_1]} & \cdots & \frac{\partial V_{0,1}}{\partial [E_n]} \\
\vdots & \ddots & \vdots \\
\frac{\partial V_{0,m}}{\partial [E_1]} & \cdots & \frac{\partial V_{0,m}}{\partial [E_n]}
\end{bmatrix} [S]_0^{-1}
$$

(6)

We combine Eqs. 5 & 6 to relate the vector of substrate cleavage, $V_0 = [V_{0,1}, \cdots, V_{0,m}]$ to the catalytic efficiencies $C$, the initial substrate concentration $[S]_0$, and the vector of specific proteases present in the reaction $E = [E_1, E_2, \cdots, E_n]$:

$$V_0^T = [S]_0 CE^T$$

(7)

We determine $C$ using mixtures of individual enzymes and substrates as described above. The dimensions of $C$ depend on the number of substrates used in the experiments and the number of enzymes considered. Both of these parameters can be customized to fit the given application. Once $C$ has been identified, Eq. 7 can readily be solved for $E$, allowing the activities of specific enzymes ($E$) to be deconvolved from non-specific cleavage signatures ($V_0$).

Various methods can be applied to solve Eq. 7. In this work, we implement a non-negative least squares algorithm combined with inference sensitivity analysis. We employ sensitivity analysis to quantitatively determine robustness to experimental error and to tune inference sensitivity and specificity. PrAMA inference involves three main procedures once $V_0$ and $C$ have been measured. First, we perform a bootstrapping scheme of randomly generating an ensemble of 1000 cleavage vectors $V_0^s$. Sampling is from a log-normal distribution with $\mu = V_0$ and a standard deviation representative of the average variance between the experimentally observed and PrAMA expected cleavage rates obtained from PrAMA validation sets of data. Second, we use least squares to solve Eq. 7 for every $V_0^s$ in the
sampling ensemble, and compute the mean and standard deviation of the ensemble inference results $E$. In some cases, we added artificial error to $C$ for each iteration of the bootstrapping scheme, representative of experimentally observed parameter uncertainty. This additional process did not significantly improve PrAMA inference, however, and was excluded unless stated otherwise.

As the third step, we apply a robustness filter to the inference results to tune specificity. This filter, termed the $\sigma_T$ threshold, roughly defines specific protease activities as significant if they are inferred in more than a certain percentage of the ensemble of inferences. We scale $\sigma_T$ as a fraction of the inference standard deviation that is subtracted from the mean inference value. For example, setting $\sigma_T = 1.0$ roughly defines protease activity as significant if observed in at least 84% of the ensemble inference results.

Results

Characterization of the non-linear kinetic model

PrAMA infers specific protease activity levels from panels of kinetic cleavage measurements using FRET-based polypeptide substrates (see Fig. 1 for a schematic & illustration of the procedure). The first steps of PrAMA involve determining the kinetic parameters $V_0$ and $C$ from time-lapse fluorimetry data (see Methods). To establish these two parameters, we employ a “depletion-decay” kinetic model of substrate cleavage that elaborates on linear M-M kinetics to account for first-order photobleaching decay and substrate depletion. Typical raw time-course fluorimetry output for a mixture of enzymes and substrates can be fit by both the linear and decay-depletion models reasonably well (Fig. 2A). However, the often subtle non-linearity of the data can reveal significant differences in the underlying kinetics. For the time-course in Fig. 2A, the cleavage rate $V_0$ inferred from the decay-depletion model is roughly 50% greater than that inferred when using the linear approximation. For a systematic comparison of the two models, we inferred trypsin activity from time-lapse fluorimetry measurements across 2–3 orders of magnitude in enzyme concentration, using both the linear and decay-depletion models (Fig. 2B). With log-log scaling, the $R^2$ coefficient of determination for the decay-depletion model in Fig. 2B is 0.9, compared to only $R^2 = 0.25$ for the linear model. Disparity in inference accuracy lies almost entirely at the extremely high and low enzyme concentrations, where substrate depletion and photobleaching, respectively, become most significant. In effect, the decay-depletion model increases the range over which protease activities can be quantitatively and accurately measured by over an order of magnitude.

Several factors make photobleaching a significant issue in this work. We conduct protease activity assays using fluorescein-based FRET substrates over long (often > 5hrs) time scales. Fluorescein is relatively sensitive to photobleaching, and long time scales further amplify photo-sensitivity effects. In this application we typically read fluorescence every 15–30 mins and observe resultant first-order decay constants ($k_d$ values) as high as $10^{-4} \text{s}^{-1}$.

Computational simulations using the decay-depletion model demonstrate how significantly decay can influence the observed fluorescence (Fig. 2C). Our results indicate that even the small amount of photobleaching incurred with infrequent plate-reader measurements may result in a several-fold decrease in fluorescence after hours have elapsed.

PrAMA Construction

PrAMA requires the explicit measurements of the entries of matrix $C$, which characterize the catalytic efficiencies with which individual enzymes cleave FRET-substrates, before specific enzyme activities can be deconvolved from complex reaction mixtures. The dimensions of $C$ can be customized depending on the experimental application, and Fig. 2D
depicts one possible configuration involving 18 FRET-substrates, 4 ADAMs, and 10 MMPs. In this figure, catalytic efficiencies range from $10^3 M^{-1} s^{-1}$ to $10^6 M^{-1} s^{-1}$. Although we successfully measured cleavage rates below this range, such low signals typically have high error and ultimately have low impact on PrAMA inference. We hierarchically biclustered the elements of C with a Euclidean distance metric and average linkage, without mean-centering or variance normalizing, and with optimal leaf ordering. Several clear patterns emerge from this clustering, as indicated by the dendrograms flanking the array (Fig. 2D). MP clustering somewhat recapitulates DNA sequence based phylogenetic profiling of the enzyme families. For example, ADAMs partition from the MMPs, and closely related MMPs such as the gelatinases (MMP2 & MMP9) cluster together. The substrates form three distinct clusters: substrates with greater specificity towards ADAMs (cyan on the dendrogram); substrates with greater specificity towards MMPs (red); and substrates cleavable by both MMPs and ADAMs (blue, green to a lesser extent). More than anything, however, C explicitly indicates the lack of selectivity among these substrates. Hence, this parameter matrix underscores the need for a deconvolution process in extracting specific protease activities from FRET-substrate measurements, and C ultimately becomes integral to this task.

**A compendium of cleavage signature measurements**

We performed roughly 4000 experiments using a variety of enzyme combinations, buffers, and substrates to measure, validate, and test the parameters and principles of PrAMA inference. We conducted a wide array of PrAMA experiments whereby panels of FRET-substrates were used to measure the cleavage signature $V_0$ of various enzyme mixtures, including roughly 50 single enzyme mixtures, 30 double enzyme mixtures, and 10 triple enzyme mixtures. We tested several buffers, enzyme concentrations, enzyme combinations, and have presented the $V_0$ values corresponding to these reaction conditions in Fig. 3. All experiments and inferred cleavage signatures $V_0$ are available in the online supplementary material. We hierarchically biclustered the reaction conditions according to their mean-centered and variance-standardized cleavage signatures $V_0$, using Euclidean distance, average linkage, and optimal leaf ordering. Clustering can often serve as an effective tool to group and/or classify objects. In this application, hierarchical clustering successfully groups over 75% of individual MMPs with themselves, based on their $V_0$ observed at different concentrations and buffers (Fig. 3A). Clustering analysis becomes more difficult to interpret in multi-enzyme mixtures, yet some patterns still emerge (Fig. 3B–C). For example, $V_0$ signatures from mixtures containing ADAM enzymes form two main clusters, corresponding to reactions with and without MMP present (Fig. 3C). Nevertheless, simple hierarchical clustering inadequately classifies such complicated enzyme mixtures, motivating a more effective inference methodology to ascertain individual enzyme activities.

**PrAMA classifies individual MPs**

We first tested the ability of PrAMA to identify an individual enzyme based on its cleavage signature observed at one concentration and/or buffer compared to another. To demonstrate, we constructed a $16 \times 5$ parameter matrix C describing the cleavage of substrates 1–16 by MMPs 1,2,3,7, and 8 at {0.4, 0.1, 0.9, 0.7, 1.0} nM, respectively, in MMP buffer. We used these parameters to analyze cleavage signatures $V_0$ from the same enzymes at an order of magnitude lower concentration. Results indicate PrAMA accurately infers the specific MMP based on its cleavage signature, with an average total cross-reactivity of 11% (Fig. 2E). As a second demonstration, we used the same parameter matrix C to infer cleavage signatures corresponding to MMPs 1–8 in MEBM buffer rather than MMP buffer, again at different concentrations. Results for this analysis are even better: 4/5 PrAMA experiments show zero cross-reactivity, and one has 13% cross-reactivity between MMP3 and MMP7 (Fig. 2F).
PrAMA inference strengths and weaknesses

We analyzed several properties of the parameter matrix $C$ in order to \textit{a priori} predict which MMPs PrAMA can accurately infer with high specificity. We transform $C$ into a model covariance error matrix $R_M$ that describes inference uncertainty as a function of data uncertainty, $R_D$, which we directly measure from the variance of replicate experiments. $R_M$ is mathematically defined as the following:

$$R_M = (C^T R_C C)^{-1} \quad (8)$$

The $R_M$ corresponding to the $16 \times 10$ parameter matrix $C$ characterizing the cleavage of substrates 1–16 by 10 MMPs reveals both absolute (Fig. 4A) and relative (Fig. 4B) amounts of model uncertainty. $R_M$ can reflect various types of experimental error depending on its construction. $R_M$, in its form described above, emphasizes multiplicative experimental error and does not \textit{a priori} make expectations regarding the concentrations of particular proteases. Fig. S1 shows a transformed $R_M$ that emphasizes additive error, which we experimentally observe to be generally much less significant than multiplicative error.

The diagonal elements of $R_M$ represent on-target model uncertainties, while off-diagonal elements indicate off-target model error. To emphasize the relative amounts of on- and off-target uncertainty, we subtract the diagonal elements of $R_M$ from their respective rows to produce a “relative” model uncertainty matrix, $R_M^r$ (Fig. 4B). Large positive values in $R_M^r$ indicate the potential for high cross-reactivity in PrAMA inference. For example, the $R_M^r$ rows for MMPs 9, 12, and 13 have large elements corresponding to MMPs 3 and 7. This suggests that signals from MMPs 9, 12, and 13 are likely to be mistakenly inferred as MMPs 3 and 7. We experimentally tested such cross-reactivity by performing PrAMA inference on MMP signals using different $C$ configurations. We tested all combinations of MMPs considered by $C$, and performed PrAMA to infer MMP activity from individual enzyme mixtures (Fig. 4C). Results indicate that indeed MMPs 9, 12, and 13 have high cross-reactivity with other MMPs. MMPs 1, 2, 3, and 7 are inferred with the greatest specificity. Encouragingly, results suggest that inference cross-reactivity is relatively independent of the number of MMPs considered, past a certain point. For most MMPs, there is hardly any increase in average cross-reactivity when increasing the number of MMPs considered from 6 to 10. For MMP13, the effects of high pairwise uncertainty become diluted when more MMPs are considered, and total cross-reactivity actually decreases. To test cross-reactivity as a function of experimental variability, we simulated PrAMA by inferring MMP activity from cleavage signatures generated from the columns of $C$, but with increasing artificial amounts of multiplicative error added to the simulated cleavage signatures. Fig. 4D shows the average results from 1000 iterations of these simulations for MMPs 1 and 10, when all MMPs are considered in the $C$ parameter matrix. In agreement with $R_M$ and $R_M^r$, MMP1 has higher on-target error, while MMP10 has high cross-reactivity. \textit{A priori} analysis of $R_M$ can suggest potential protease inhibitors to add or biophysical separation techniques to apply in order to eliminate the number of MMPs considered in a given sample. Both on- and off-target error significantly decrease when MMPs 1 and 10, which according to $R_M$ have relatively low cross-reactivity, are the only two proteases considered in PrAMA inference. Ultimately this analysis (a) reveals the potential need for additional FRET-substrates with certain specificities, (b) suggests which MMPs can be accurately and simultaneously measured in a given sample, and (c) suggests the potential use of inhibitors or supplementary experimental methods to achieve greater inference resolution.
Inference sensitivity analysis improves PrAMA accuracy

Experimental error propagates through PrAMA inference in a complex manner. Consequently, we perform a bootstrapping sensitivity analysis to directly account for observed experimental error, gauge its effect on PrAMA inference, and to tune PrAMA specificity/sensitivity (see Methods). Experimental replicates of C and $V_0$ have an average standard deviation of 20%. Experimental variance is a mix of both additive and multiplicative error, and standard deviation drops to roughly 10% at protease activity levels above $10^8 M^{-1}s^{-1}$. In general, our results suggest that correct PrAMA inference is more robust to experimental and/or artificial noise than incorrectly inferred enzyme activities (i.e. false positive results). As an example, we inferred MMP activity from a cleavage signature $V_0$ corresponding to a reaction that contained MMP8, but with increasing synthetic multiplicative sampling error applied to the observed $V_0$ (Fig. 5A). In this instance, MMP8 does not have the highest average inferred MP activity when synthetic sampling error is high. Nonetheless, PrAMA infers MMP8 activity with the greatest consistency compared to the other MPs considered. We developed a robustness threshold, termed the $\sigma_T$ threshold, to take advantage of this general observation. Total cross-reactivity of the inferred MMP activity is a function of (a) the experimental and/or synthetic sampling error of the parameters $V_0$ and C, and (b) the $\sigma_T$ threshold. For PrAMA inference of the single-enzyme mixtures involving MMPs 1–8, total cross-reactivity can be totally eliminated by applying the appropriate $\sigma_T$ threshold, even when the standard deviation of the applied Gaussian multiplicative error approaches 100% (Fig. 5B). As another example, the $\sigma_T$ threshold can completely eliminate as much as 170% cross-reactivity in the inference of individual MMPs 9, 10, 12, and 13 (Fig. 5C–D).

PrAMA inference of MP mixtures

We tested the ability of PrAMA to infer specific protease activities from cleavage signatures $V_0$ observed from mixtures of two and three MPs. A priori inspection of the model error matrix $R_M$ indicates that MMPs 1, 2, 3, 7, and 8 show the lowest cross-reactivity with each other, so we initially considered only these 5 enzymes. We analyzed 20 single-enzyme mixtures, 12 two-enzyme mixtures, and 5 triple-enzyme mixtures involving these 5 MMPs. For all mixtures, we did not consistently observe statistically significant deviation between the observed cleavage signatures $V_0$ and the expected cleavage patterns based on PrAMA assumptions (e.g., see Eq. 7). Figs. S2–S12 show raw time-lapse fluorimetry data, inferred & expected cleavage signatures $V_0$, and PrAMA inference results for several of these enzyme combinations.

For each mixture, we define MP activity as significant if inferred at levels above a defined $\sigma_T$ threshold. If that enzyme is actually present in the reaction mixture, then we label the inference for that specific MP as “true positive.” Receiver-operator characteristic (ROC) curves then summarize the total PrAMA inference results (Fig. 6A). Tuning the $\sigma_T$ threshold moves inference results along the ROC curve to adjust the true positive and false positive rates. Inference accuracy, defined as the ratio (true positives + true negatives) / (total positives + total negatives), is maximally above 90% for the single and double enzyme mixtures. For the triple enzyme mixture, maximum accuracy is roughly 80%. We explored an alternative bootstrapping method in this work (see Methods), but found PrAMA results to be robust to the algorithm variation (Fig. S13). Fig. 6D shows PrAMA inference results for the double-enzyme experiments with increasing levels of $\sigma_T$. Previous information regarding MP interactions, co-expression, and cellular localization partly informed the selection of MP mixture compositions. For example, mixtures involving membrane-bound ADAM enzymes also included MMP14, which is the only MMP we analyzed that is membrane-bound with a transmembrane domain. We also included MMP2 in these mixtures, as previous work indicates MMP14 activates MMP2 at the cell surface [46]. We
analyzed 5 double-enzyme and 10 single-enzyme mixtures involving ADAM10, ADAM17, MMP2, and MMP14 (Fig. 6B, E). For these enzymes, maximum PrAMA inference accuracies for both the single and double enzyme mixtures are roughly 90%. Lastly, we analyzed 20 single-enzyme, 6 double-enzyme, and 2 triple-enzyme mixtures involving MMPs that show high cross-reactivity, namely MMPs 9, 10, 12, 13, and 14. Even for these enzymes, PrAMA inference performs with a maximum accuracy of roughly 90% for all single, double, and triple enzyme mixtures. This positive result indicates that the cross-reactivity discussed in Fig. 4 can be effectively attenuated by applying an appropriate \( \sigma_T \) threshold.

We performed PrAMA inference on mixtures containing various concentrations of MMP7 to ascertain PrAMA’s ability to quantitatively infer MMP activity, in addition to simply inferring whether or not an enzyme is present (Fig. 6F). We used 16 substrates and considered 10 MMPs (i.e., constructed a 16 × 10 matrix \( C \)) in this analysis, and tested 7 concentrations ranging from 0.01nM to 1nM. In all cases, PrAMA inferred MMP7 activity with 100% specificity. Furthermore, PrAMA detected quantitative differences in protease activity with high accuracy. \( R^2 = 0.98 \) for a log-log plot that describes inferred MMP7 activity as a function of its actual concentration. PrAMA has less success in quantifying absolute differences in activity among multiple MPs (Fig. S14A–B), in part due to the fact that the relationship between MMP concentration and observed protease activity \( V_0 \) is enzyme-specific and can deviate from linearity (Fig. S14C, Fig. S15). In general, we observe the recombinant MMPs employed in this work to be less efficient at higher concentrations. Enzyme concentration effects on proteolytic activity may be due to issues such as non-specific protein adsorption and aggregation. To test this hypothesis, we added increasing concentrations of Brij 35 to the reaction buffer (Fig. S16A). Although Brij can decrease proteolytic efficiency, our results suggest that Brij improves assay linearity perhaps by decreasing non-specific aggregation at higher enzyme concentrations (Fig. S16B), which has been observed for other secreted proteins [47]. Even when nonlinear relationships between MMP concentration and observed protease activity \( V_0 \) exist, PrAMA inference does not seem to distort these relationships (Fig. S14C). Consequently, quantitative comparisons of individual protease activities from one experimental sample to another can still be accurately made.

**PrAMA with unknown background protease activity**

In many potential applications, PrAMA will not be able to explicitly account for all protease activities in the parameter matrix \( C \). In this work we account for up to 14 MPs simultaneously, and more substrates and enzymes can be potentially included in the PrAMA for future applications. Nevertheless, some biological samples may contain unknown proteinases that are also capable of cleaving the FRET-substrates. Robustness to these unknown proteinases is a crucial property of PrAMA. To test this, we applied PrAMA inference to enzyme mixtures with known “background” protease activity that is not explicitly accounted for in the parameter matrix \( C \). For example, we constructed a 16 × 2 parameter matrix \( C \) to infer MMP9 & MMP10 activities. We tested PrAMA inference on 5 enzyme mixtures that contained MMP9 and/or MMP10, as well as at least one additional MP that was unaccounted for in \( C \) (Fig. 7A). We repeated this process for MMPs 1–8 (3 mixtures) and ADAMs 10 & 17 (3 mixtures). All three sets of analyses performed roughly as well as the PrAMA inference results where all protease activity was explicitly accounted for in \( C \). PrAMA inference of ADAMs 10 & 17 yielded a maximum accuracy of 100%, although statistical significance was modest (p=0.17) due to the small sample size (3 mixtures) and low inference dimensionality (only 2 enzymes). Statistical significances of the other two PrAMA results were greater (MMP9 & 10, p<0.1; MMPs 1–8, p<0.001).
We also used PrAMA to infer protease activity over a background of conditioned media from the breast cancer cell line MDA-MB-231 (Fig. 6F). We added recombinant, active MMP7 to supernatant collected 12hrs after stimulating cells with EGF and the inflammatory cytokine TNFα. We considered 10 MMPs in the parameter matrix C, and ultimately were able to identify MMP7 protease activity with 100% specificity. PrAMA did not detect any additional MMP activity in these samples.

Augmenting PrAMA with specific protease inhibitors

The accuracy and specificity of PrAMA can be bolstered by using protease inhibitors in conjunction with traditional PrAMA methods. Adding inhibitors to solutions of active proteases may be appropriate when specificity and accuracy are considered more important than non-invasiveness. In this work, we present an example of how PrAMA can be combined with inhibitors. First, we measured the cleavage profile $V_0$ of a mixture containing ~0.5nM MMP3 & MMP7 and ~0.05nM MMP9 in MMP buffer using substrates 1–16. Second, we measured the cleavage rate $V_0$ of substrate-1 when the mixture had an added 100nM MMP-9 inhibitor ($IC_{50} = 5nM$). The decrease in observed cleavage rate caused by adding the inhibitor, divided by the previously known catalytic efficiency $C_{ij}$ and substrate concentration, produced an inferred MMP9 concentration within 20% of the actual concentration: $[E] = V_0/([S]C_{ij}) \approx 0.04nM$. Based on this, we subtracted the expected MMP9 component of the cleavage signature from the total signature $V_0$ observed with no inhibitor and performed PrAMA inference on the remaining non-MMP9 component. For this example, PrAMA achieves correct inference of all three enzymes only when incorporating information gleaned from using the inhibitor (Fig. 7B). Without this information, PrAMA fails to infer MMP9 without including several false-positives.

PrAMA inference of live-cell response to phorbol ester stimulation

We applied PrAMA to a well-studied set of wildtype, ADAM10−/−, and ADAM17−/− MEF cell lines in order to validate the approach in a live-cell context. Fig. 8A depicts the increased substrate cleavage observed in response to PMA stimulation for four of the total seven substrates used in this example. Within 2hrs, PMA causes a statistically significant increase in substrate cleavage for at least one substrate in all three cell lines (Fig. 8B): 4/7 substrates significantly increased cleavage with WT cells; 3/7 increased with ADAM10−/− cells; and substrate 15 increased cleavage with ADAM17−/− cells. In some cases, we observe more statistically significant changes in cleavage when including measurements at later time points. Substrates 1, 9, and 15 significantly increase cleavage in ADAM17−/− cells when assessing cleavage 8hrs after PMA stimulation (p<0.05). From these measurements it would be difficult to attribute changes in substrate cleavage to particular MPs without PrAMA. We assess protease activity by using a parameter matrix C that considers the presence of five MPs (Fig. 8C). We define the observed cleavage vector $V_0$ as the change of cleavage rate in response to PMA stimulation, using all seven substrates. PrAMA inference indicates that PMA stimulates significant ADAM17 activity (Fig. 8D). In all three cell lines, PrAMA did not detect a significant increase in MMP2, MMP14, or ADAM10 activity. Results indicate that substrate cleavage and subsequent PrAMA are sensitive to treatment with the metalloproteinase inhibitor GM6001 (Fig. S17). As further validation, PrAMA infers ADAM17−/− cells to have 90% less ADAM17 activity than WT. Remaining ADAM17 signal in ADAM17−/− cells likely arises from other proteases (e.g., ADAM9) with similar substrate selectivity. PrAMA results that show ADAM17 to be activated in response to PMA agree with multiple other reports in the literature [19, 20, 25]. This work ultimately complements these previous reports by observing specific ADAM17 activity in a non-invasive, real-time manner, without resorting to pharmacological or genetic perturbations.
Optimal substrate selection

Various logistical constraints may exist that limit the number of substrates available to be used for PrAMA in certain applications. To address this issue, we implemented a common optimal design criterion for selecting substrates so as to maximize PrAMA accuracy. The determinant of the covariance error matrix $R_M$ is one metric describing the volume of inference uncertainty. Minimizing this volume, which is equivalent to maximizing the determinant of its inverse and minimizing the condition number, ultimately reduces model uncertainty and optimizes PrAMA inference accuracy [48]. Using the following function, we optimally selected subsets of the total 18 FRET-substrates to perform various PrAMA inferences:

$$\min \det(R_M) = \max \det(C^T R_o C)$$ (9)

We tested the substrate selection strategy on PrAMA inference of mixtures involving MMPs 1, 2, 3, 7, and 8, where only those 5 enzymes are considered in the parameter matrix $C$ (Fig. 9A–E). In general, the impact of optimal substrate selection increases as the number of substrates decreases. Surprisingly, optimal substrate selection allows PrAMA to use the theoretically minimum number of substrates, equal to the number of MMPs considered in the parameter matrix $C$, without significantly impacting inference accuracy. PrAMA inference of double-enzyme mixtures containing ADAM10, ADAM17, MMP2, and MMP14 maintains an accuracy indicated by the area under the ROC curve (AUROC) of roughly 0.9 even as the number of substrates decreases by nearly 50% (Fig. 9F). In general, the optimal combination of substrates depends on which MPs are being analyzed by PrAMA, and the optimal substrates combination for distinguishing ADAM activities is distinct from the optimal combination for distinguishing activities of MMPs 1–8.

Discussion

Existing techniques used to study MPs each have advantages and disadvantages. Zymography is one of the oldest and most common MP activity assays, and a variety of zymographic techniques exist to measure the activities of diverse proteases and their inhibitors [28, 49, 50]. Most zymographic techniques involve SDS-PAGE electrophoresis, which prevents continuous real-time measurement (and generally disrupts non-covalent MP complexes). In situ zymography, often applied to frozen tissue sections, allows for the observation of localized protease activity [27]. However, in most cases the substrates used for in situ zymography (e.g., gelatin) are readily cleaved by a variety of MPs and measurements consequently lack specificity. Recently a variety of methods have been developed to observe the proteolytic degradation of endogenous MP substrates. For example, mass spectrometric techniques can quantify hundreds of proteins that have freshly cleaved amide bonds within complex biological samples [26]. Problems involving the complex relationship between proteases and their substrates make it difficult to accurately and non-invasively infer the contributions of specific MPs to the global patterns of endogenous substrate degradation. Yet another recently developed method, activity based probes (ABPs), support the direct and specific measurement of diverse protease activities [29, 30, 51]. Like typical zymography substrates, however, ABPs can act on a broad range of related proteases. Therefore, assays involving ABPs face a trade-off between invasiveness and specificity. For specific protease identities to be ascertained, biological samples analyzed with ABPs can be resolved by size through electrophoresis.

Synthetic polypeptide protease substrates have been developed for an increasingly wide range of enzymes. Within the last few years several FRET-substrates have been designed with some specificity, thereby supporting their application in complex biological samples...
Nevertheless, cross-reactivity with closely related MPs and distantly-related, but much more non-specific, proteases can still complicate the interpretation of FRET-substrate activity assays. As an example, several FRET-substrates with some specificity for ADAM17 have recently been developed with a sequence based on the ADAM17 cleavage site on pro-TNFα [31, 32, 52, 53]. Multiple recent reports employ these ADAM17 FRET-substrates, even though they have documented cross-reactivity with related MPs [33, 54]. At least six MPs have been recognized to cleave endogenous pro-TNFα, in some cases at the same site [32, 55, 56]. Such non-specificity complicates interpretation of the observed FRET-substrate cleavage, especially when comparing multiple correlated MP activities in the same biological sample [54]. Several MPs cleave many of the same synthetic and endogenous substrates that ADAM17 cleaves, suggesting the repertoire of ADAM17 substrates could be a subset of the repertoire for more promiscuous MPs (e.g., MMP14) or non-specific proteases like plasmin [57]. At least to some degree, this situation is conceivable not just for ADAM17 but for a variety of MPs, and would make identifying truly specific substrates impossible.

Although MPs have been extensively studied for decades, no method yet exists to assay multiple protease activities in real-time with high specificity and non-invasiveness. One explanation partly accounting for this fact is that the ubiquitous regulatory interactions, diverse substrates, and distinct roles played by closely related MPs have only recently become fully appreciated. Both MMPs and ADAMs engage in regulatory networks controlled by cyclical feedback interactions. For example, ADAMs participate in an autocrine positive feedback loop in mammary epithelial cells: EGFR transactivation stimulates Erk activity, which in turn stimulates ADAM shedding of EGF ligands, further activating EGFR [59]. In this situation, common methods of ascertaining the influence of ADAM activity on EGFR signaling, such as by applying a protease inhibitor or siRNA treatment, can both disrupt the underlying feedback interactions and potentially create compensatory reactions whereby closely related ADAMs modify their activity to accommodate perturbations [15, 24, 25, 58]. As another example, many MMPs activate themselves and one another. Such interactions can create positive feedback interactions that allow, for example, an initiating MMP activation event to trigger further protease activation [60]. We predict non-invasive, multiplexed, real-time, and specific measurements of MP activity will be critical towards understanding the complex regulatory mechanisms underlying MP networks.

We anticipate that PrAMA should have broad applicability in protease biology. FRET-substrates have been extensively used for high-throughput inhibitor screening with individual purified enzymes. PrAMA would allow inhibitor screening to be performed in more complex enzyme mixtures and biological samples, and could be adapted for high-throughput in vitro functional assays of inhibitor activity. As discussed above, PrAMA is well suited for network-level analysis of in vitro protease activity, and PrAMA can scale up and down in scope depending on the particular application. At the most basic level, PrAMA could use multiple FRET-substrates in tandem to bolster the specificity of an activity measurement for even a single protease. In other words, the parameter matrix C could be as small as (2 substrates x 1 enzyme). PrAMA can capture protease activity on a variety of time-scales, depending on the particular application. We demonstrate high sensitivity measurements that are made over the course of >5hrs, and live-cell measurements can detect significant differences in cleavage within 30min. Dynamic measurements on this short time-scale can be relevant for detecting rapid post-translational protease activation, while longer time-scale measurements have relevance, for example, to phenotypic responses that are downstream of transcriptional changes. Soluble FRET-substrates can be directly applied to both live-cells and cell lysate for protease activity measurement [31, 54]. Our initial experiments show that PrAMA can operate by adding individual yet distinct FRET-
substrates to live-cells in a multi-well format. Furthermore, FRET-substrates with distinctive excitation/emission spectra may be simultaneously combined in the same solution for PrAMA of a single biological sample. FRET-substrates have been tethered directly to 3D substrata such as collagen [61], providing localized measurement of protease activity. For simultaneously analyzing many protease activities, the mathematical framework behind PrAMA can be applied to microarrays of peptides, for instance, that contain hundreds or thousands of FRET-peptide substrates. Furthermore, the principles behind PrAMA are readily extendable to other classes of enzymes, such as caspases and cathepsins. FRET-based protease substrates have been successfully applied to measuring in vitro caspase activation. Like MPs, however, individual caspases have overlapping substrate specificity and it can be difficult to interpret which specific caspase has become activated [62]. Lastly, PrAMA inference has many potential uses involving clinical samples. For example, simultaneous measurement of multiple protease activities in patient fluid samples or biopsies could reveal mechanistic insight and/or identify activity-based markers of disease state for diagnostic/prognostic use. Ultimately, this work presents an integrated mathematical and experimental framework that can be adapted and extended to a broad range of applications. We have demonstrated various methods of a priori analyzing how best to design PrAMA experiments, whether it be through choosing optimal substrates, identifying which proteases can be specifically measured with the available substrates, or understanding how to account for experimental variability.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABP</td>
<td>activity based probe</td>
</tr>
<tr>
<td>ADAM</td>
<td>a disintegrin and metalloproteinase</td>
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<tr>
<td>B</td>
<td>background signal</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>$C_{i,j}$</td>
<td>catalytic efficiency for the $i$th substrate and $j$th enzyme</td>
</tr>
<tr>
<td>C</td>
<td>catalytic efficiency parameter matrix</td>
</tr>
<tr>
<td>Cha</td>
<td>cyclohexylalanyl</td>
</tr>
<tr>
<td>Dabcyl</td>
<td>4-(4-dimethylaminophenylazo)benzoyl</td>
</tr>
<tr>
<td>$[E]$</td>
<td>enzyme concentration</td>
</tr>
<tr>
<td>E</td>
<td>vector of enzyme activities</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>$F_0$</td>
<td>peak fluorescence from positive control</td>
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$F_{obs}$ observed fluorescence from product formation
$F_p$ fluorescence from product formation
Fam 5-carboxyfluorescein
FRET fluorescence resonance energy transfer
Homophenylalanyl
$k_{cat}$ turnover number
$k_{cat}/K_m$ catalytic efficiency
$k_d$ photobleaching decay constant
$K_m$ Michaelis-Menten constant
M-M Michaelis-Menten
MEBM Mammary Epithelial Basal Medium
MMP matrix metalloproteinase
MP metalloproteinase
PrAMA Proteolytic Activity Matrix Analysis
$R_M$ model covariance error matrix
$R_M^r$ relative model covariance error matrix
$R_D$ data uncertainty covariance matrix
$[S]_0$ initial substrate concentration
$[S]$ substrate
$\sigma_T$ significance of inference threshold
$T_0$ lag time
TIMP tissue inhibitor of metalloproteinase
TNF$\alpha$ tumor necrosis factor-alpha
$V_0$ initial rate of substrate cleavage
$V_0^s$ bootstrapping sample ensemble of multiple $V_0$

References

Figure 1. PrAMA overview
Blue indicates PrAMA development & construction, red indicates PrAMA implementation, and grey indicates experimental preparation & procedure.
Figure 2. Modeling protease cleavage kinetics and specificity signatures

(A) A typical time-lapse fluorimetry output for a single enzyme (0.5nM MMP10) and substrate 13 in MMP buffer. The data is fit with both linear and decay-depletion kinetic models. The decay-depletion model almost perfectly overlays the data. (B) Inferred kinetic rates ($V_0/[S]_0$) of trypsin cleaving substrate 7 over a range of concentrations. (C) Decay-depletion model simulations of substrate cleavage using the following parameters: $F_0 = 10^4$ FLU, $k_{cat}/K_M = 10^5$ M$^{-1}$s$^{-1}$, $[E]=1$nM, $[S]=10$μM. (D) Hierarchical biclustering of observed cleavage efficiencies for various ADAMs and MMPs. Cleavage parameters were averaged over several concentrations. (E,F) PrAMA inference of individual MMPs at different concentrations (E, ~0.5nM vs. ~0.05nM) and buffers (F, MMP minimal media vs. MEBM). Each column represents an individual PrAMA experiment. The abscissa indicates the actual enzyme present, and the ordinate indicates the inferred MMP present. Each PrAMA output (each column) is normalized to have a total signal of 1.
Figure 3. A compendium of cleavage signatures from purified proteases and protease mixtures
Hierarchical biclustering organizes reaction mixtures by their corresponding cleavage signatures, which consist of cleavage rates (sec$^{-1}$) of substrates (shown in columns) by various protease mixtures (shown in rows). Within each signature $V_0$, the Z-score indicates deviation from the mean cleavage rate, following variance-standardization. Three arrays accompany each subplot A–C. The left array describes the buffer (indicated by white) that corresponds to each cleavage signature, aligned by row with the other two arrays. The middle array describes MMP concentrations, and corresponds by row with the adjacent cleavage signatures. Experiment groupings are as follows: (A) all single MMP experiments, (B) all single and mixture MMP experiments, and (C) all single and mixture experiments involving ADAMs.
Figure 4. Parameter matrix error analysis
(A) Model error covariance matrix $R_m$. (B) Relative model error matrix, $R'_m$. (C) Median off-target PrAMA inference error as a function of the number of MMPs considered in the parameter matrix, averaged over all possible combinations of MMP subsets. (D,E) Average target and off-target inference error as functions of the synthetic measurement error, when all MMPs (D) or only MMPs 1 & 10 (E) are considered in the parameter matrix. (C–E) Parameter matrix C was constructed using enzymes at ~0.5nM. $\sigma_T = 0$ for all results here. (C) Cleavage signatures were obtained at ~0.05nM.
Figure 5. Robustness thresholds filter off-target inference

(A) Inference results for cleavage signatures obtained using MMP 8 at ~0.05nM and parameters obtained at ~0.5nM. Increasing amounts of multiplicative error was added to cleavage signatures, randomly sampled from a normal distribution with standard deviations of $\sigma = \{33\%, 67\%, 100\\%\}$. (B) Total cross-reactivity in inference results for cleavage signatures obtained using MMPs 1, 2, 3, 7, and 8 at ~0.5nM and parameters obtained at ~0.05nM. Cross-reactivity is undefined at high sampling error and $\sigma_T$ thresholds, when no signal falls above $\sigma_T$. (C, D) PrAMA inference of MMPs 9, 10, 12, and 13 at different concentrations (~0.5nM and ~0.05nM) before (C) and after (D) applying 30% sampling error and threshold $\sigma_T = 2$. Each column represents an individual PrAMA experiment. The abscissa indicates the actual enzyme present, and the ordinate indicates the inferred MMP present. Each PrAMA output (each column) is normalized to have a total signal of 1.
Figure 6. PrAMA inference of enzyme mixtures

ROC curves describing PrAMA accuracy for single and mixture enzyme experiments involving (A) MMPs 1, 2, 3, 7, and 8; (B) ADAMs 10, 17, and MMP 2, 14; (C) MMPs 9, 10, 12, 13, and 14. (D, E) Heat maps indicating PrAMA inference results using different $\sigma_T$ thresholds. Each column corresponds to a different enzyme mixture, and rows indicate which enzymes are considered in the parameter matrix. Each PrAMA output (each column) is normalized to have a total signal of 1. D corresponds to the double-enzyme ROC curve in A. E corresponds to the double-enzyme ROC curve in B. (F) PrAMA inference results (ordinate) of MMP7 at different actual concentrations (abscissa). Inference was performed using substrates 1–16 in MMP buffer (black) and conditioned media (red).
Figure 7. Using PrAMA with background protease activity and protease inhibitors
(A) The figure legend indicates which MMPs are considered in the parameter matrix. The corresponding ROC curves describe PrAMA inference accuracy for mixtures that contain MMPs and ADAMs that are both considered and ignored in the parameter matrix. (B) Inference for a triple-enzyme mixture involving MMP3, MMP7, and MMP9, with or without invoking information from an MMP9 inhibitor. For inference with inhibitor, p<0.001.
Figure 8. Live cell inference of MP activity
PrAMA was conducted using three cell lines (WT, ADAM10−/−, and ADAM17−/− MEFs) and 7 total substrates, tracking substrate cleavage up to two hours after adding substrate. (A) Time-lapse fluorimetry for 4 of the 7 total substrates used in this experiment. (B) Inferred substrate cleavage rates ($V_0/[S]_0$), corresponding by row to the time-courses shown in A. Stars indicate $p<0.05$, comparing between cleavage rates for the control and stimulated conditions. (C) Parameter matrix used in this experiment, with each column divided by its Euclidean norm. (D) PrAMA inference results for the increased activity caused by PMA stimulation, using significance threshold $\sigma_T=1.4$. No significant increase in MMP2, MMP14, or ADAM10 activity was detected at this threshold. For MMP9 and ADAM17, all inferred differences were statistically significant ($p<0.05$). For all subplots, error bars indicate standard deviation of three biological replicates.
Figure 9. Optimal substrates selection improves PrAMA accuracy

(A,B) ROC curves describing PrAMA accuracy for double-enzyme experiments involving MMPs 1,2,3,7, and 8, where PrAMA uses a worst (A) or best (B) subset of the substrates, as defined in the text. (C–F) Area under the ROC curve (AUROC) as a function of the number of best (black) or worst (red) substrates, for various sets of PrAMA experiments: single (C), double (D), and triple (E) enzyme mixtures involving MMPs 1,2,3,7, and 8; (F) double-enzyme mixtures involving ADAMs 10 and 17.
Figure 10.