

Red-shifted fluorescent proteins monitor enzymatic activity in live HT-1080 cells with fluorescence lifetime imaging microscopy (FLIM)

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Summary

Membrane type 1 matrix metalloproteinase (MT1-MMP) is a membrane-tethered collagenase primarily involved in the mechanical destruction of extracellular matrix proteins. MT1-MMP has also been shown to be upregulated in several types of cancers. Many coordinated functions of MT1-MMP during migration and invasion remain to be determined. In this paper, live cells from the invasive cell line HT-1080 were imaged using an intracellular Förster resonance energy transfer-based biosensor specific for MT1-MMP; a substrate specific for MT1-MMP was hybridized with the mOrange2 and mCherry fluorescent proteins to form the Förster resonance energy transfer-based sensor. The configuration of the biosensor was determined with fluorescence lifetime-resolved imaging microscopy using both a polar plot-based analysis and a rapid data acquisition modality of fluorescence lifetime-resolved imaging microscopy known as phase suppression. Both configurations of the biosensor (with or without cleavage by MT1-MMP) were clearly resolvable in the same cell. Changes in the configuration of the MT1-MMP biosensor were observed primarily along the edge of the cell following the removal of the MMP inhibitor GM6001. The intensities highlighted by phase suppression correlated well with the fractional intensities derived from the polar plot.

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Introduction

Matrix metalloproteinases (MMPs) are a family of enzymes that process and degrade extracellular matrix proteins. Many diseases, including several cancers, are associated with elevated MMP expression (Nakada *et al.*, 1999; Zhai *et al.*, 2005). An MMP known as membrane type 1 matrix metalloproteinase (MT1-MMP) localizes at the leading edge of tumours (Hofmann *et al.*, 2003) and promotes local invasiveness. Although the regulation of invasiveness by MT1-MMP is not well understood, it is known that the requirement of MT1-MMP for invasiveness depends on the density and type of extracellular matrix proteins surrounding the cell (Wolf *et al.*, 2003; Sabeh *et al.*, 2004; Hotary *et al.*, 2006; Zaman *et al.*, 2006).

Previous studies on cells also indicate that MT1-MMP can regulate and promote the cell's migration. These regulatory mechanisms of MT1-MMP are linked to the phosphorylation of its cytoplasmic tail (Nyalendo *et al.*, 2007; Nyalendo *et al.*, 2008), shedding of external proteins (Kajita *et al.*, 2001; Endo *et al.*, 2003) and its internalization at the membrane (Uekita *et al.*, 2001). However, many unanswered questions still exist about MT1-MMP's role regulating and coordinating the cell's behaviour during migration and invasion.

To determine MT1-MMP's roles specifically during invasiveness, attempts to study the activity of MT1-MMP and other MMPs in live cells have included a variety of sensors (Golubkov *et al.*; Ouyang *et al.*, 2008; Ouyang *et al.*, 2010; Packard *et al.*, 2009). Studies using collagen-based

fluorescent sensors to monitor cancer cells migrating in three-dimensional matrices indicate that MMPs were likely active primarily along the leading edge of the cell (Packard *et al.*, 2009). An intracellular biosensor developed by Ouyang *et al.* monitored the activation of MT1-MMP following stimulation by the epidermal growth factor (Ouyang *et al.*, 2008; Ouyang *et al.*, 2010). The active MT1-MMP enzyme, visualized with this intracellular biosensor, was present primarily along the periphery of HeLa cells. In this case however, the MT1-MMP enzyme was exogenously introduced into the HeLa cells.

In this paper, we examine the activity of the same intracellular MT1-MMP biosensor (Ouyang *et al.*, 2008; Ouyang *et al.*, 2010) in the established invasive (fibrosarcoma) cell line, HT-1080 (Rasheed *et al.*, 1974). HT-1080 cells endogenously express the MT1-MMP enzyme. As a result of Förster resonance energy transfer (FRET), the fluorescence emission of this biosensor varies depending on the extent of its interaction with MT1-MMP (Förster, 1948a; Förster, 1948b; Förster, 1951; Clegg, 1992). The MT1-MMP biosensor can monitor the activity of MT1-MMP throughout the entire membrane simplifying the process of making correlations to specific subcellular structures and organelles.

Recently, the MT1-MMP biosensor has been modified to contain the mOrange2 and mCherry fluorescent proteins (along with the pre-existing substrate specific for MT1-MMP) to improve the quality of its detection (Shaner *et al.*, 2004; Shaner *et al.*, 2008; Ouyang *et al.*). The long wavelength fluorescent proteins can be excited and emit light at wavelengths greater than 550 nm. For live cell imaging this offers advantages, including the ability to image without the contribution of autofluorescence typically encountered with the cyan and yellow fluorescent proteins. Live cells and tissues are also not as readily damaged by longer excitation wavelengths.

The configuration of the MT1-MMP biosensor was assessed on a pixel-wise basis using fluorescence lifetime imaging microscopy (FLIM) (Lakowicz & Berndt, 1991; Gadella *et al.*, 1993; Clegg, *et al.* 1996; Schneider & Clegg, 1997; Periasamy & Clegg, 2010). In this work, FLIM was performed in the frequency domain. To image the cells rapidly, images of cells transfected with the MT1-MMP biosensor have also been collected with a variant of FLIM called phase suppression. Phase suppression selectively highlights intensities in the image from each configuration of the biosensor with high contrast (Lakowicz & Cherek, 1981; Lakowicz & Cherek, 1982; Lakowicz & Berndt, 1991; Lakowicz *et al.*, 1992; Clegg *et al.*, 1996; Schneider *et al.*, 1997; Holub *et al.*, 2000; Eichorst *et al.*, 2011). It requires only two exposures of the sample, which, depending on the brightness of the image, can result in acquisition times of less than a second. This is considerably shorter than for a typical full-field FLIM measurement, where multiple image exposures are necessary. As reported previously using purely steady-state imaging, changes in the configuration of the biosensor and also

the development of protrusive structures along the cell's perimeter can require minutes (Ouyang *et al.*; Ouyang *et al.* 2008). Therefore, phase suppression improves the time resolution for following the dynamic activity of the MT1-MMP biosensor.

Localizing and following MT1-MMP's activity in time is crucial to correlate the cell's dynamics with MT1-MMP activity. Currently, we have monitored the spatio-temporal activation of MT1-MMP's activity following the removal of the MMP inhibitor GM6001 in live HT-1080 cells. With FLIM using both the polar plot and phase suppression a clear correlation between both methods of analysis was demonstrated.

Background/materials and methods

Measurements of FRET efficiency

The biosensor's configuration can be followed by measuring the FRET efficiency (E) (Clegg, 1992; Förster, 1948a, 1948b, 1951). FRET efficiency of a single donor and acceptor sample is related to the distance (r) between the donor and acceptor fluorophores by Eq. (1),

$$E = \frac{1}{1 + \left(\frac{r}{R_o}\right)^6} \quad (1)$$

The Förster radius (R_o) is the distance where ($E = 0.5$). R_o depends on the donor's quantum yield, the acceptor's extinction coefficient, the mutual orientation of the donor's and acceptor's transition moments and also on the overlap (in wavelength or frequency) of the acceptor's absorption spectrum with the donor's emission spectrum. A biosensor having two configurations is described by two different FRET efficiencies. If $0.6 \leq \frac{r}{R_o} \leq 1.5$, the FRET efficiency can accurately report on the configuration of the biosensor. Keeping the distances between the donor and acceptor within these limits, where the sensitivity of changes in FRET as a function of separation distance is maximum, is especially important when a biosensor is used in live samples, where more extensive time averaging can cause photolytic damage.

The use of long wavelengths is advantageous for FRET-based studies because the overlap integral has a fourth power wavelength dependency over the range of wavelengths applied in the integral. As reported for the mOrange/mCherry pair (Goedhart *et al.*, 2007), this increase in the value of the overlap integral inherent with longer wavelengths results typically in higher R_o . Hence, longer wavelength fluorophores can improve the sensitivity in the detection of the biosensor by providing larger changes in FRET efficiency at greater separation distances between the donor and acceptor. At this time, only a limited number of reports have been published applying long wavelength fluorescent proteins for FRET-based applications (Goedhart *et al.*, 2007).

A variety of methods exist to quantify FRET. Measuring the fluorescence lifetime (average time a molecule remains in the excited state) is a direct method to determine FRET efficiencies, particularly for imaging applications. The intrinsic lifetime of the acceptor does not change as a result of FRET; however, in the presence of an acceptor, the measured lifetime of the donor is shortened (Birks, 1970; Clegg, 1992; Valeur, 2002; Lakowicz, 2006). The measured time dependence of the acceptor's emission can be affected, and this variation can also be used to assess the extent of FRET in the system (Birks, 1970; Lakowicz & Balter, 1982a, 1982b; Hanley & Clayton, 2005; Chen & Clegg, 2011).

Fluorescence lifetime imaging microscopy

Frequency domain

In the time domain, the fluorescence response to a very short pulse of excitation (e.g. a delta function) is expressed as a sum of exponential decays

$$I(t) = \sum_i a_i e^{-\frac{t}{\tau_i}}. \quad (2)$$

We refer to Eq. (2) as the fundamental fluorescence response. The factor (a_i) is the species fraction and is proportional to the concentration of the fluorescent species (i).

In the frequency domain, the fluorescence lifetime is measured by exciting the sample with a repetitively intensity-modulated light. Any repetitive waveform can be used to modulate the intensity of the excitation source. Repetitive waveforms can be decomposed by a Fourier series analysis into independent sinusoidal components; thereby the intensity of the exciting light can be expressed and analysed as a weighted sum of independent sinusoids. If the repetitive frequency is $f_E = \frac{\omega_E}{2\pi}$, then the fundamental component of the excitation light modulation can be expressed as Eq. (3).

$$E(t) = E_o + E_\omega \cos(\omega_E t + \phi_E). \quad (3)$$

The intensity-modulated fluorescence emission $F(t)$ is the convolution of the fundamental fluorescence response with the waveform of the excitation. For the excitation waveform of Eq. (3) the resulting fluorescence is a sinusoid oscillating at the same frequency as the excitation waveform (Eq. 4).

$$F(t) = F_o + F_\omega \cos(\omega_E t + \phi_E - \phi_F). \quad (4)$$

The fluorescence emission $F(t)$ is demodulated and shifted in phase relative to the excitation. Demodulation refers to a decrease in the amplitude of the sinusoidal oscillation of a waveform relative to the time-averaged (DC) value of the signal. For a single fluorescing sample with a single lifetime, the demodulation (M) and phase shift (ϕ_F) are related to the lifetime of the sample according to Eqs (5) and (6), where

$$\tau_{MOD} = \tau_\phi.$$

$$M = \frac{F_\omega/F_o}{E_\omega/E_o} = \frac{1}{\sqrt{1 + (\omega_E \tau_{MOD})^2}} \quad (5)$$

$$\phi_F = \tan^{-1}(\omega_E \tau_\phi). \quad (6)$$

Multicomponent lifetimes

If the sample exhibits multi-exponential decay (multicomponent lifetimes), the phase shift and demodulation do not refer to the decay constant of any individual component. Therefore, Eqs (5) and (6) cannot be used to determine the lifetime of any single component (for multicomponent signals, $\tau_{MOD} > \tau_\phi$). The lifetimes of most fluorescent proteins exhibit multicomponent lifetimes.

The relationships between the constituent lifetimes and the measured phase shift and demodulation can be derived by applying Fourier analysis to the normalized fundamental fluorescence response (Eq. 2). If the excitation waveform is repetitive, both the excitation waveform as well as the intensity-modulated fluorescence can be written as the sum of orthogonal basis functions (sines and cosines) oscillating at a set of frequencies $\omega_{k,E} = k\omega_E$. The Fourier coefficient of each k th frequency component represents that frequency's effective sinusoidal contribution to the actual excitation and fluorescence signals. By summing over all the lifetime components, the normalized measured demodulation (M_k) and phase shift ($\phi_{k,F}$) of each k th frequency component can be written as,

$$M_k = \sqrt{\left(\sum_i \frac{\alpha_i}{1 + (\omega_{k,E} \tau_i)^2}\right)^2 + \left(\sum_i \frac{\alpha_i \omega_{k,E} \tau_i}{1 + (\omega_{k,E} \tau_i)^2}\right)^2} \quad (7)$$

$$\phi_{k,F} = \tan^{-1} \left(\frac{\sum_i \frac{\alpha_i \omega_{k,E} \tau_i}{1 + (\omega_{k,E} \tau_i)^2}}{\sum_i \frac{\alpha_i}{1 + (\omega_{k,E} \tau_i)^2}} \right). \quad (8)$$

In Eqs (7) and (8) the term (α_i) is the fractional contribution to the measured steady-state intensity of the fluorescent species having the lifetime (τ_i) (referred to as fractional intensity). The relationship between the fractional intensity and the species fraction is shown in Eq. (9).

$$\alpha_i = \frac{a_i \tau_i}{\sum_i a_i \tau_i}. \quad (9)$$

Characterizing a sample with a multicomponent lifetime either in the time or frequency domain can be time consuming. In the frequency domain, measurements of the phase shift and demodulation at many frequencies combined with iterative fitting are required to solve for the individual lifetime components (Gratton & Limkeman, 1983; Gratton *et al.*, 1984).

Polar plots

The application of the polar plot is a direct method to visualize and analyse the measured demodulation and phase shift without lengthy fitting procedures (Jameson *et al.*, 1984; Clayton *et al.*, 2004; Redford & Clegg, 2005; Digman *et al.*, 2008). The x - y coordinates of the polar plot are,

$$x = M \cos(\phi_F) \quad (10)$$

$$y = M \sin(\phi_F). \quad (11)$$

When measuring in the frequency domain, the phase shift and the demodulation are measured quantities. Therefore, the polar plot is constructed directly from the data.

If all the contributing fluorescent components are directly excited by the excitation light (i.e. no fluorescent component is excited by an excited state reaction, such as in FRET), points on the polar plot from fluorescent species with a single lifetime will reside on the perimeter of a semicircle, whereas those characterized by multicomponent lifetimes will have polar coordinates located inside the semicircle. The analysis of a directly excited fluorescent species on the polar plot can be accomplished with vector addition. For example, the polar coordinate of a sample composed of several discrete lifetimes is the sum of the constituent polar coordinates weighted by their corresponding fractional intensities, as shown in Eqs (12) and (13).

$$x = \sum_i \alpha_i M_i \cos(\phi_i^F) \quad (12)$$

$$y = \sum_i \alpha_i M_i \sin(\phi_i^F) \quad (13)$$

The variables (M_i) and (ϕ_i^F) refer to the demodulation and phase shift of the fluorescent component with lifetime τ_i , where

$$M_i = \frac{1}{\sqrt{1 + (\omega_E \tau_i)^2}} \quad (14)$$

$$\phi_i^F = \tan^{-1}(\omega_E \tau_i). \quad (15)$$

In Eqs (14) and (15) we consider only a single frequency.

The polar plot is a convenient, rapid and model-free presentation of the time-dependent fluorescence emission. Quantitative information about component lifetimes such as species fraction and fractional intensity can be derived from the plot (Clayton *et al.*, 2004; Redford & Clegg, 2005; Digman *et al.*, 2008). Likewise, even without determining component lifetimes, the polar plot graphically shows changes between different populations of fluorophores. We use this analysis to study the two configurations of the MT1-MMP biosensor (each having a distinct FRET efficiency).

Frequency mixing

We acquire lifetime-resolved images with a full-field, frequency-domain, homodyne FLIM system (Lakowicz & Berndt, 1991; Gadella *et al.*, 1993; Schneider & Clegg, 1997). In homodyne detection, the gain of the detector (an intensifier placed in front of a charge-coupled device camera) is modulated at the same frequency as the modulation of the excitation light's intensity. The resulting signal $S(\phi_G - \phi_E)$ recorded by the detector is time independent and depends on the difference in phase between the excitation light (ϕ_E) and the detector's gain (ϕ_G)

$$S(\phi_G - \phi_E) = F_o G_o \left(1 + \frac{F_o G_o}{2 F_o G_o} M \cos(\phi_G - \phi_E + \phi_F) \right). \quad (16)$$

For the data acquisition, the charge-coupled device camera averages the full-field images at a series of phase differences ($\phi_G - \phi_E$) over a full period to determine the demodulation (M) and phase shift (ϕ_F). The modulation frequency is between 10 and 100 MHz to detect lifetimes in the range of nanoseconds (Lakowicz & Berndt, 1991; Gadella *et al.*, 1993; Clegg *et al.*, 1996; Schneider & Clegg, 1997).

A typical exposure time required to collect a single-phase image (an image $S(\phi_G - \phi_E)$) collected at a specific value of ($\phi_G - \phi_E$) is on the order of 1–200 ms; this depends on the brightness of the image (longer integration times increase signal-to-noise). In general, unless considerations such as photobleaching, object motion or kinetics set limits on the integration times, the exposure time is chosen to use the majority of the dynamic range of the intensifier and camera. The total time required to obtain a full FLIM image depends on the number of phase images acquired over a full period. In this study, the phase stability of the detection was confirmed before and after each FLIM phase image. Usually eight FLIM images, each corresponding to different detector phase settings, were acquired. Therefore, depending on the exposure time for different samples and phase checks, several seconds elapsed between complete consecutive FLIM images.

Phase suppression/enhancement

Phase suppression uses images acquired at paired homodyne phase settings to remove the intensity contributions from fluorescence components with specific lifetimes. This technique, first applied in single channel cuvette-based frequency domain lifetime measurements, (Veselova *et al.*, 1970; Veselova & Shirokov, 1972; Lakowicz & Cherek, 1981; Gratton *et al.*, 1984) has been subsequently applied to frequency domain FLIM (Lakowicz & Berndt, 1991; Lakowicz *et al.*, 1992; Clegg *et al.*, 1996; Schneider *et al.*, 1997; Holub *et al.*, 2000; Eichorst *et al.*, 2011). Phase suppression removes the intensity from a fluorescent species with a specific phase shift (i.e. lifetime) by subtracting two phase images where

the intensities from the fluorescent species to be suppressed are equal. As applied in this paper, the two images selected to suppress the intensity from a fluorescent species in an image are chosen to be 180° out of phase along the homodyne curve of intensity values describing the species to be suppressed. The intensity contributions from fluorescent species with phase shifts differing from the phase shift of the suppressed species (different lifetimes) will be retained partially in the difference image. Although the two images are often chosen at detector phases that are 180° apart, there are a variety of positions along the sinusoid $S(\phi_G - \phi_E)$ that are useful to suppress a specific lifetime. The benefit of phase suppression is that fluorescent species with different lifetimes can be distinguished with high contrast, and spatially mapped with only two exposures of the sample.

Image analysis

Wavelet analysis

Images acquired on the homodyne FLIM system were denoised using an algorithm customized for the Gaussian noise in our detection system (Willet & Nowak, 2004; Spring & Clegg, 2009). Following the denoising step, the background was removed from each image by applying multiresolution analysis to each of the eight primary phase images acquired for lifetime imaging and to the images acquired for phase suppression (Mallat, 1989; Buranachai *et al.*, 2008). For background removal, images were decomposed with the *Sym8* wavelet using the Discrete Wavelet Transform in MatLab (Mathworks, Natick, MA, USA).

Fractional intensity mapping

Many images of the HT-1080 cells in this paper are presented with a colour code. The colour in each pixel was determined by the positions of each pixel on the polar plot relative to the centres of the intact and cleaved biosensors. More specifically, the colour of each pixel depends on the contribution of each configuration (intact or cleaved) of biosensor to the steady-state intensity measured in that pixel. The configuration which contributes the largest fractional contribution to the total measured steady-state intensity, determines the colour in the pixel (red = intact biosensor, green = cleaved biosensor). However, the steady-state intensities of both configurations of the biosensor are similar because our emission filter accepts emission from both the donor and acceptor. Therefore, the fractional intensities are also estimates of the normalized concentration of each configuration of the biosensor (species fraction). With this analysis, spatial features of the biosensor's activity were observed and validated by analysis directly on the polar plot.

The fractional contributions to the steady-state intensity of the cleaved and intact biosensor at each pixel are determined

from the polar plot coordinates according to the following matrix calculation.

$$\begin{bmatrix} x_{\text{meas}} \\ y_{\text{meas}} \end{bmatrix} = \begin{bmatrix} x_{mOr2} & x_{\text{Bios}} \\ y_{mOr2} & y_{\text{Bios}} \end{bmatrix} \begin{bmatrix} \alpha_{mOr2} \\ \alpha_{\text{Bios}} \end{bmatrix}. \quad (17)$$

In Eq. (17), the terms (x_{mOr2}) , (y_{mOr2}) , (x_{Bios}) and (y_{Bios}) are the coordinates of the cleaved (mOr2) and intact biosensor (Bios) on the polar plot. The corresponding measured data points are (x_{meas}) and (y_{meas}) . The calculated contributions of each configuration (cleaved = mOr2, intact = Bios) of the biosensor to the measured steady-state intensity at a pixel are (α_{mOr2}) and (α_{Bios}) .

The polar coordinates of the two configurations of the MT1-MMP biosensor $((x_{mOr2}), (y_{mOr2}), (x_{\text{Bios}})$ and $(y_{\text{Bios}}))$ were determined by measuring a series of HT-1080 cells transfected with the biosensor. The positions on the polar plot assigned to each configuration of the biosensor were computed from bivariate Gaussian fits applied to the FLIM data from live cells. Before representing the distribution of FLIM data on the polar plot by two-dimensional Gaussian functions, the pixels belonging only to the cells were selected segmented (Otsu, 1979) after the suppression of background and noise by wavelets. The threshold was adjusted so that the majority of the cell body from each image was selected for the set of cells examined during this analysis. Following the segmentation, the pixels were binned to form a three-dimensional histogram on the polar plot (Spring & Clegg, 2009).

The data from the HT-1080 cells, which were not treated with the GM6001 inhibitor, were fitted with a double Gaussian bivariate fit (Fig. S1 and Table 1). The centres of the two Gaussian functions representing the cleaved and intact biosensor were found and are shown with their respective uncertainties in Table 1.

A single bivariate Gaussian fit applied to the images of live cells containing the MT1-MMP biosensor but pretreated with the GM6001 inhibitor. GM6001 prevents the cleavage of the biosensor. After nine cells were analysed, the fitting of these polar coordinates resulted in a single Gaussian distribution centred at a polar coordinate of $[0.764 \pm 0.0774, 0.380 \pm 0.0566]$. The above polar coordinates derived from the control HT-1080 cells (not pretreated with GM6001) were used to represent the positions of the two configurations of the biosensor on the polar plot applied in this paper.

FLIM system

The excitation light from a 532-nm laser (World Star Tech, Toronto, Canada) was intensity modulated by a Pockels Cell (Conoptics Inc., Danbury, CT, USA). The modulation frequency for the lifetime measurements was 40 MHz. All samples were imaged on a Leica DMIRB microscope (Leica, Germany). The emission was collected through a dichroic and emission filter combination passing 560–640 nm (Omega

Table 1. Gaussian fitting on the polar plot applied to HT-1080 cells not treated with GM6001: The polar coordinates determined using the demodulation and phase shifts taken from the pixels within the HT-1080 cells containing the MT1-MMP biosensor were binned in a three-dimensional histogram on the polar plot. The results from fitting the data with two Gaussian functions are shown as a function of the number of cells examined. The centres of the fitted distributions, the corresponding standard deviations and fit parameters are shown.

#Cells	x_1 Centre	y_1 Centre	x_1 SD	y_1 SD	x_2 Centre	y_2 Centre	x_2 SD	y_2 SD	R^2
5	0.6791	0.4565	0.1119	0.08974	0.3948	0.2626	0.2337	0.2639	0.964
6	0.6729	0.4421	0.1181	0.1028	0.8293	0.3625	0.04082	0.03304	0.928
7	0.6712	0.4352	0.1139	0.09389	0.8201	0.368	0.04275	0.03359	0.92
8	0.6713	0.4312	0.1136	0.09526	0.8156	0.3701	0.04479	0.03244	0.923
9	0.6749	0.4356	0.1058	0.08486	0.8147	0.3702	0.04436	0.03253	0.912
10	0.6754	0.4272	0.1057	0.08717	0.8132	0.3674	0.04475	0.03215	0.918

Optical, Brattleboro, VT, USA). The detection system consisted of an intensifier (Kentech, England) modulated at high frequency and charge-coupled device camera (QImaging, Surrey, BC) positioned at the output port of the microscope. The charge-coupled device camera collected images by performing a 2×2 binning resulting in images of 678×518 pixels. The live cells were imaged using a 100x/1.4NA oil immersion objective (Leica). All *in vitro* measurements on the FLIM system were performed with a 40x/0.75NA air objective (Leica). The lifetime reference for all lifetime measurements was 1 μ M Rhodamine 6G (Aldrich, Milwaukee, WI, USA) in water [lifetime = 4.11 ns (Hanley *et al.*, 2001)].

Cell culture

HT-1080 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) and transfected with Lipofectamine 2000 (Invitrogen). Following transfection, the cells were starved in 0.5% Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA, USA) for 36–54 hours before imaging. The cells were incubated with 20 μ M GM6001 (Merck, Germany) for 12–18 h before imaging. During imaging, the GM6001 was washed out 5–6 times using CO₂ independent media (Invitrogen) supplemented with 0.5% FBS.

Protein purification and characterization

The mOrange2/mCherry MT1-MMP biosensor was purified by Nickel chelation after being expressed with an N-terminal His₆ tag in *Escherichia coli* (Wang *et al.*, 2005). Steady-state spectra were collected on a PC1 fluorometer (ISS Inc., Champaign, IL, USA) in a solution at approximately 1 μ M of the purified biosensor. The excitation wavelength was 532 nm and the emission was collected at 1 nm increments from 555 to 750 nm. The recorded spectra were corrected for lamp fluctuations, background, the additional volume of trypsin (Invitrogen) for cleaving the biosensor and for emission-side corrections. Magic angle polarizer conditions were applied. The spectra were decomposed with linear spectral unmixing (Tsurui *et al.*, 2000) with reference spectra provided by Roger

Tsien's Lab (Tsien). Lifetime measurements were carried with a solution of 1 μ M of purified biosensor placed on a glass-bottomed Petri dish (Cell E&G, Houston, TX, USA). Lifetime data were collected both before and after the biosensor was cleaved by trypsin using the filters described previously as well as with an additional 570 nm (± 10 nm) bandpass filter (Omega Optical).

Results

In vitro measurements of the mOrange2/mCherry MT1-MMP biosensor

The MT1-MMP biosensor containing the mOrange2 and mCherry fluorescent proteins was examined *in vitro* with both steady-state and time-resolved fluorescence measurements. The solutions of biosensors measured in this section were expressed, extracted and purified from competent bacterial cells. Trypsin was used to cleave the biosensor for this characterization. The ability of trypsin to cleave the substrate of the biosensor can be seen clearly in the polyacrylamide gel (Fig. 1D). Only the cleaved components with masses between 25 and 37 kD remain in the centre band; in the left-most band, the intact biosensor contained proteins with masses between 50 and 75 kD.

Time-resolved fluorescence of the biosensor was measured on the homodyne FLIM instrument at a modulation frequency of 40 MHz, exciting the sample with a 532-nm laser. Three fluorescent species (mOrange2, mCherry undergoing FRET and directly excited mCherry) were detected through a dichroic and emission filter combination passing 560–640 nm (Fig. 1C). When both configurations of the biosensor were measured (intact and cleaved) through this dichroic/filter combination, the polar coordinate of the biosensor upon cleavage moved toward longer lifetimes (Fig. 1A). The movement of the polar coordinate after cleavage toward longer lifetimes (Fig. 1A) suggests a loss of FRET.

To measure the mOrange2 protein in both the intact and cleaved biosensor, a bandpass filter passing only the emission of mOrange2 (570 ± 10 nm) was inserted before the detector.

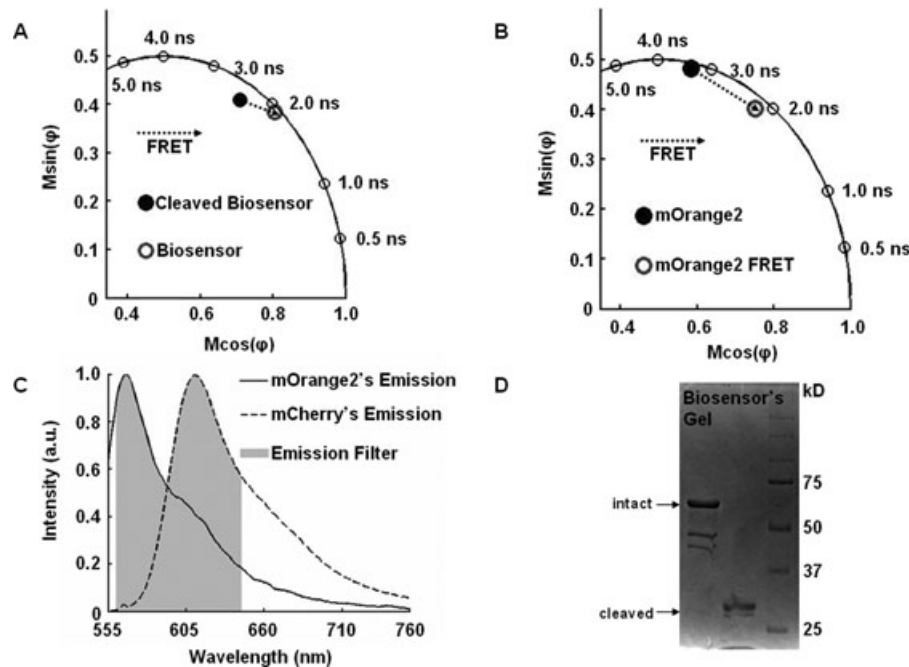


Fig. 1. Time-resolved *in vitro* measurements of a purified solution of the MT1-MMP biosensor: (A) The polar coordinates of the MT1-MMP biosensor were measured before and after the biosensor was cleaved by trypsin. Measurements were taken at a modulation frequency of 40 MHz through the emission filter shown in (C). (B) The phase shift and demodulation of the mOrange2 was also measured on both configurations of the biosensor at an emission wavelength of 570 ± 10 nm. When measured and plotted on the polar plot at a modulation frequency of 40 MHz, there is a definite movement of the polar coordinate toward longer lifetimes after cleavage of the biosensor. (C) The normalized emission spectra of mOrange2 and mCherry are displayed along with the filter pass of the emission filter highlighted in (A) (and used for the live cell measurements). (D) Separate protein solutions of the intact biosensor and the biosensor cleaved by trypsin were examined in a polyacrylamide gel. The leftmost lane contains the intact biosensor and the centre lane contains the cleaved biosensor. The far right lane is the indicator lane displaying the reference masses.

In both configurations of the biosensor, the mOrange2 had a multicomponent lifetime distribution (Fig. 1B). However, the cleavage of the biosensor resulted in the polar coordinate for mOrange2 emission moving from near 2 ns towards a region on the polar plot between 3 and 4 ns (Fig. 1). This movement of the polar coordinate to a region of longer lifetimes after the cleavage of the biosensor also confirms the presence of FRET before the cleavage.

Steady-state measurements were performed at an excitation wavelength of 532 nm to monitor changes in mOrange2's and mCherry's emission spectra before and after cleavage of the biosensor's substrate. In these experiments, a solution of purified biosensor was measured in a fluorometer both before and after the substrate of the biosensor was cleaved by trypsin. Spectral unmixing was applied using reference spectra from Roger Tsien's Lab (Tsien) to isolate the spectral contributions from mOrange2 and mCherry (Fig. 2). There was a clear increase in the fluorescence emission from mOrange2 following the cleavage of the biosensor (Figs. 2A and 2B). In parallel, the emission from mCherry decreased indicating the absence of FRET in the system (Figs. 2A and B). The change in the spectrum of mCherry is only

discernable after spectral unmixing because of bleed-through from mOrange2's emission.

Spatial features of the MT1-MMP biosensor in HT-1080 cells

HT-1080 cells transfected with MT1-MMP biosensor were imaged with FLIM, using the emission filter shown in Figure 1(C) and a 532-nm laser for excitation. To examine the cleaved biosensor, the HT-1080 cell in Fig. 3 was imaged after incubation with the GM6001 inhibitor for 12–18 h. The measured polar coordinates in the regions of interest selected within the cell (white squares in Fig. 3A and the polar plots in Fig. 3E) remained near 2 ns, indicating that the biosensor was mostly intact. Likewise, the fractional intensities, depicted by the colour coding in Figures 3(B), (C) and (D), indicated that the biosensors within the cell were intact (red shading = intact biosensor, green shading = cleaved biosensor).

When cells were not incubated with the GM6001 inhibitor, the locations of the polar coordinates (Fig. 4A and E) and the mapping of fractional intensities (Fig. 4B–D) indicated that both configurations of the biosensor were present in the cells. Pixels from the regions of interest in Figure 4(A) are projected

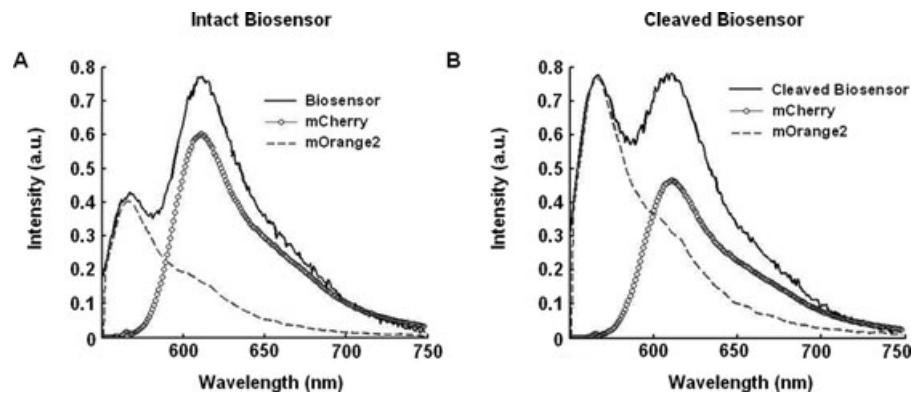


Fig. 2. Steady-state measurements taken on a purified solution of the MT1-MMP biosensor: (A) The emission spectrum of the intact MT1-MMP biosensor was measured with an excitation wavelength of 532 nm. Spectral unmixing was applied to resolve the contributions of the mOrange2 and mCherry separately. (B) The emission spectrum of the cleaved biosensor was also measured at an excitation wavelength of 532 nm. After the biosensor was cleaved, there is a definite loss in emission from the mCherry and an increase in fluorescence emission from the mOrange2 relative to (A) indicating a loss of FRET.

on the polar plots in Figure 4(E); two separate lifetime pools near 2 ns and 3 ns are observed. The fractional intensities within the HT-1080 cells not treated with the GM6001 inhibitor (Fig. 4B–D) showed that the intact biosensor was contained primarily in the brighter region near the nucleus (red shading in Fig. 4B and D), whereas the cleaved biosensor appeared mostly around the cell’s periphery (green shading in Fig. 4C and D).

Phase suppression applied to examine live cells

In these experiments, two phase images were acquired and subtracted to suppress intensity from the intact biosensor,

and separately two different phase images were acquired and subtracted to suppress the intensity from the cleaved biosensor. The two selected phase images were subtracted so that positive intensities remained in areas of the image containing fluorescent species that were not suppressed; that is, if the intact biosensor was suppressed, then the cleaved biosensor would appear as a positive value in the difference image. Any negative values in areas of the difference images where intensity from a specific fluorescent species was being suppressed were because of noise or precision of the phase setting. Only positive values are shown in the phase suppression images. During these experiments, the HT-1080 cells transfected with the biosensor were excited with a

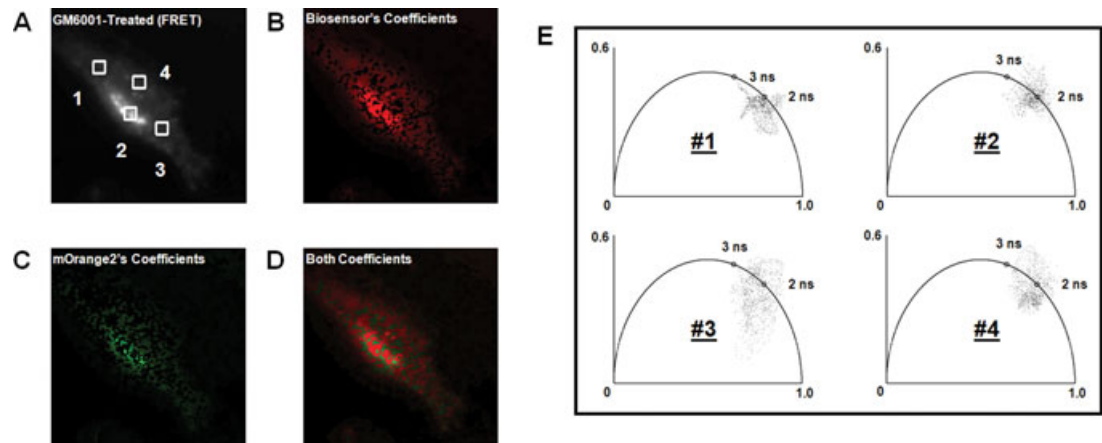


Fig. 3. HT-1080 cell treated with the GM6001 inhibitor: (A) An intensity image of an HT-1080 cell taken during a homodyning FLIM experiment is presented with four regions of interest highlighted in white squares. (B) An IMD (intensity-masked display) image is shown where the red shading indicates where the fractional intensity of the intact biosensor is dominant relative to the cleaved biosensor. (C) In this IMD image, the areas in the cell where the fractional intensity from the cleaved biosensor (mOrange2) is dominant are shown in green. (D) When both (B) and (C) are combined, the resulting cell image shows that most of the collected intensity is being emitted from the intact biosensor as indicated by the red shading. (E) The pixels from the regions in interest in (A) are projected on the polar plot in these scatter plots. The plots indicate that there is likely a single lifetime pool near 2 ns representing the intact biosensor.

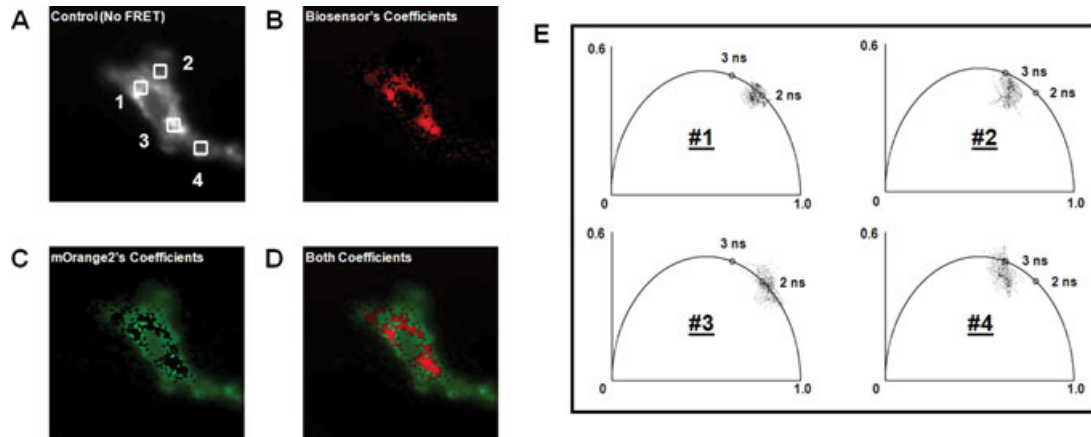


Fig. 4. HT-1080 cell not treated with the GM6001 inhibitor: (A) An intensity image of an HT-1080 is shown with regions of interests (#1–4) highlighted. (B) Red shading is presented indicating where the fractional intensity from the intact biosensor is most prevalent. (C) Highlighted in green are the areas in the cell where the fractional intensity of the cleaved biosensor (mOrange2) is dominant in the image. As shown, these areas reside mostly on the edge of the cell. (D) Images (B) and (C) were then combined to form an image where the dominant fractional intensities of both configurations of the biosensor are highlighted together. (E) These scatter plots are projections of the pixels from the regions of interest in (A) on the polar plot. Two lifetime pools between 2 ns (regions #1 and #3) and 3 ns (regions #2 and #4) are visible.

532 nm laser and the emission was collected through the filter described in Figure 1(C).

Experiments were performed to determine whether phase suppression would reliably record changes in configuration of the biosensor in the HT-1080 cells after the GM6001 inhibitor was washed out. Rhodamine 6G was used to determine accurately the phase setting of the instrument before the measurements on cells. Four phase angles were selected for the phase suppression to suppress the intensity from mOrange2 (cleaved biosensor) and separately to suppress the intensity from the intact biosensor. Full-field homodyne FLIM images were taken in parallel with the images gathered for the phase suppression. During these experiments, images were acquired before the washout of GM6001 (Fig. 5) as well as at a time approximately 30 min following the washout (Fig. 6).

As shown by red shading in Figure 5(D), before the washout of the GM6001 inhibitor, the intact biosensor's fractional intensity was dominant in the majority of the cell body. Some intensity from the cleaved biosensor appeared along the cell's edge (green shading in Fig. 5C). When the pixels from the regions of interest in Figure 5(E) were projected on the polar plots in Figure 5(F), the polar coordinates at locations within the cell body were close to 2 ns. Near the bottom of the cell (region 3 in Fig. 5E and F), the polar coordinates corresponded to lifetimes above 2 ns. When phase suppression was applied to suppress the intensity from the intact biosensor (Fig. 5A) very little intensity remained in the cell body with only a few low intensity features appearing along the cell's edge. However, when the intensity from the mOrange2 (cleaved biosensor) was suppressed (Fig. 5B) the intensity remaining in the difference image (indicative of the intact biosensor) was shown to be distributed throughout the majority of the cell's interior body.

Following the washout of GM6001, the fractional intensity around the edge of the cell was dominated by mOrange2 (cleaved biosensor) (green shading in Fig. 6C). The difference image shown in Figure 6(A), where the intact biosensor's intensity was suppressed, paralleled the trends indicated by the polar plot analysis using fractional intensities. Thus, when the intensity from the intact biosensor was suppressed, intensity in the difference image, representing free mOrange2 (cleaved biosensor), was found predominantly at the cell's edge (Fig. 6A). This latter intensity, corresponding to free mOrange2, matched well to the green regions in Figure 6(C) where the fractional intensity from the cleaved biosensor was dominant. Likewise, the intensity from the intact biosensor remained primarily within the central portion of the cell body (Fig. 6B and D). In addition, when projected on the polar plot, the pixels from the regions of interest 1 and 3 are located closer to 3 ns whereas region 2 remained closer to 2 ns (Fig. 6E and F).

The observed changes in configuration of the biosensor following the washout of the inhibitor GM6001 were also accompanied by cell spreading and expansion (Figs S2 and S3). Both before and after the removal of the GM6001, the intensity from the intact biosensor was also observed in the central region of the cell (red shading in Fig. S2D and S3D). Following the removal of the GM6001, the analysis of data collected using phase suppression (Fig. S3A and B) and the fractional intensities derived from the polar plot (Fig. S3C and D) confirmed the spreading and configurational change in the biosensor along the edge of the cell. Direct projection of the collected FLIM data on the polar plot indicated furthermore that the polar coordinates in areas near the perimeter of the cell (regions of interest #1 and #3 in Figs S2E, S2F, S3E and S3F), moved from approximately 2 to 3 ns following the washout of GM6001.

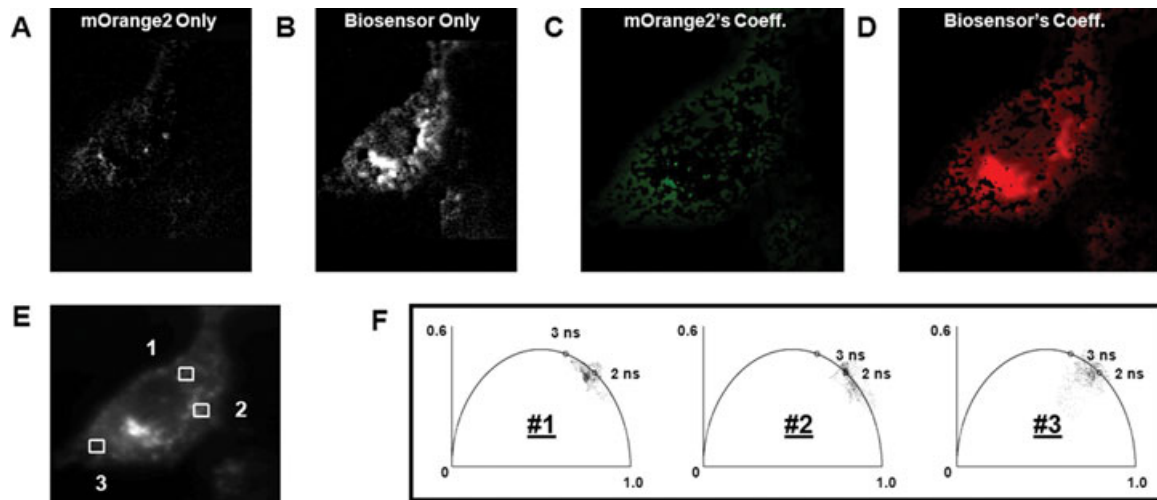


Fig. 5. Phase suppression applied to study an HT-1080 cell before the GM6001 inhibitor was washed out: (A) Phase suppression was applied to suppress the intensity from the intact biosensor in the image, leaving only intensity from the cleaved biosensor (mOrange2). (B) A set of detector phase angles was also selected to suppress the intensity from the cleaved biosensor (mOrange2). In this image, the result of that suppression strategy shows that the intact biosensor's fluorescence is being emitted from a majority of the cell's body. (C) A colour coding masked by the intensity image in (E) has been applied to the image. The green shading in the various pixels indicates where in the cell the fractional intensity of the cleaved biosensor (mOrange2) dominates the average intensity measured. (D) The pixels in which the fractional intensity is majority from the intact biosensor are coloured in red. This image has been masked by the intensity image in (E). (E) An intensity image of the cell is shown with three regions of interest indicated. (F) The pixels from each of the regions of interest in (E) are projected on the polar plot.

Discussion

The activation of the MT1-MMP biosensor was observed with both FLIM and phase suppression before and subsequent to the washout of GM6001 in live HT-1080 cells. Two lifetime

pools were observed from the FLIM measurements taken on the biosensor in live cells, indicating the presence of both intact and cleaved forms of the biosensor. The lifetime values and fractional intensities of the two lifetime pools observed in the polar plots were sufficiently dissimilar to differentiate clearly

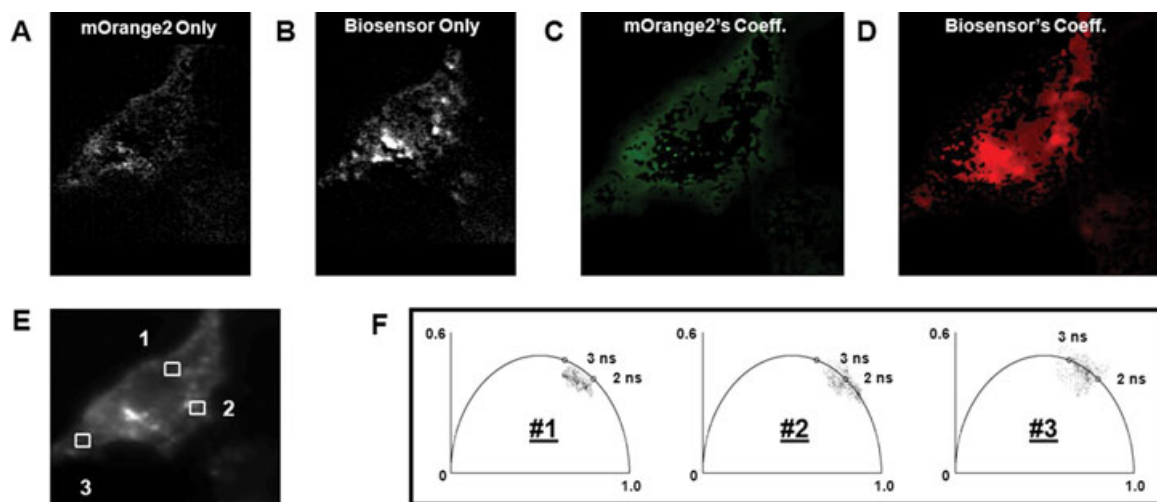


Fig. 6. Analysis of an HT-1080 cell with phase suppression after the GM6001 inhibitor was washed out: (A) Two detector phases were chosen and images were acquired to suppress the intensity from the intact biosensor. As shown, intensity remained from the cleaved biosensor (mOrange2) along the edge of the cell. (B) Phase suppression was also used to suppress the intensity of the cleaved biosensor (mOrange2). When performed, the intensity from the intact biosensor was found predominantly in the interior of the cell. (C) Overlaid on the intensity image in (E) is a colour coding describing the locations where the majority of the average intensity measured in the pixel comes from the cleaved biosensor (mOrange2). (D) Coloured in red are the pixels in which the majority of the fractional intensity is from the intact biosensor. (E) An intensity image of the cell following the washout of GM6001 is shown along with three regions of interest indicated. (F). Three polar plots are shown in which the pixels from the regions of interest in (E) are projected.

the two populations by phase suppression. The locations of intact and cleaved forms of the biosensor in the HT-1080 cells highlighted by the phase suppression closely paralleled the locations identified by the fractional intensities derived from the polar plot. Significant advantages of phase suppression are the limited exposure of the sample to light and the high contrast displayed in the difference images highlighting the two configurations of the biosensor.

In both phase suppression and polar plot-analyzed images describing the HT-1080 cells not pretreated with the MMP inhibitor and those cells for which the MMP inhibitor has been washed out, the active MT1-MMP was present along the periphery of the cell. This observation indicates that the active form of MT1-MMP has undergone correct transportation and localization to the membrane. The inactive form of MT1-MMP resides mostly in perinuclear regions; this is indicated by the lack of proteolytic activity, which is possibly the result of the pro-MT1-MMP form of the enzyme. Further work is ongoing to determine the significance of such activation patterns during the processes of migration and spreading.

This study is the first time quantitative imaging has been carried out where both configurations of the MT1-MMP biosensor can be followed in time in live metastatic cancer cells. Previous results examining the ECFP/YPet-variant of the MT1-MMP biosensor in live cells with ratiometric intensity measurements (the simple ratio of the acceptor's and the donor's fluorescence) provided qualitative information about the location of the different biosensor species in the images (Ouyang *et al.*, 2008). However, ratio-based intensity FRET measurements do not take into consideration corrections that are required to interpret the measured changes in FRET in terms of physical information, such as normalized concentrations of the intact or cleaved biosensor throughout an image (Tron *et al.*, 1984). Because the simple FRET ratio does not make any of these corrections, even large changes in the FRET ratio may correspond only to small changes in the population of the biosensor in images. This is especially true if the quantum yield of the acceptor is much greater than the donor. The FLIM experiments reported here can follow quantitatively the activity of this biosensor in live metastatic cancer cells (HT-1080) and robustly detect each configuration. The polar plot coordinates derived from the directly measurable phase shift and demodulation measurements are robust and quantitative classifiers of the biosensor's configuration, which require no corrections from the raw phase and modulation data.

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Supporting Information

Additional supporting information may be found in the online version of this paper:

Fig. S1. Gaussian fitting applied to the polar plot.

Fig. S2. HT-1080 cell exhibiting cell spreading before washout of GM6001.

Fig. S3. HT-1080 cell exhibiting spreading following the GM6001 washout.

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