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Real-time, continuous detection of maltose using bioluminescence resonance energy transfer (BRET) on a microfluidic system

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ABSTRACT

We have previously shown that a genetically encoded bioluminescent resonance energy transfer (BRET) biosensor, comprising maltose binding protein (MBP) flanked by a green fluorescent protein (GFP^2) at the N-terminus and a variant of Renilla luciferase (RLuc2) at the C-terminus, has superior sensitivity and limits of detection for maltose, compared with an equivalent fluorescent resonance energy transfer (FRET) biosensor. Here, we demonstrate that the same MBP biosensor can be combined with a microfluidic system for detection of maltose in water or beer. Using the BRET-based biosensor, maltose in water was detected on a microfluidic chip, either following a pre-incubation step or in real-time with similar sensitivity and dynamic range to those obtained using a commercial 96-well plate luminometer. The half-maximal effective concentrations (EC_{50}) were 2.4×10^{-7} M and 1.3×10^{-7} M for maltose detected in pre-incubated and real-time reactions, respectively. To demonstrate real-time detection of maltose in a complex medium, we used it to estimate maltose concentration in a commercial beer sample in a real-time, continuous flow format. Our system demonstrates a promising approach to in-line monitoring for applications such as food and beverage processing.

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1. Introduction

Förster resonance energy transfer (FRET) is a phenomenon in which a donor molecule in its excited state non-radiatively transfers energy to a proximal ground state acceptor (Forster, 1959). The most widely used form of FRET uses optical excitation of the donor molecule, i.e. fluorescence resonance energy transfer also, somewhat confusingly, known as FRET. FRET has been combined with microfluidics to realize powerful bioanalytical systems (Varghese et al., 2010). Bioluminescence-resonance energy transfer (BRET) is a form of FRET where the donor molecule is excited by a chemical reaction and energy is transferred from a chemiluminescent donor to a fluorescent acceptor (Pfleger and Eidne, 2006). In BRET, the donor fluorophore is replaced with a luciferase (Dacres et al., 2009b). We have previously shown that $BRET^2$ transduction provides superior sensitivity over FRET and $BRET^1$, for biosensors based on protease target sequences or on a bacterial periplasmic binding protein (PBP) (Dacres et al., 2009a, 2009b, 2013, 2011), albeit at the expense of relatively lower luminance. $BRET^2$ has a larger Förster distance than does classical FRET (Dacres et al., 2009c), which provides certain advantages over longer working distances (e.g. 5–10 nm). Accordingly, a $BRET^2$ system with excellent spectral separation was developed and used to estimate thrombin proteolytic activity (Dacres et al., 2009a, 2009b; Dacres et al., 2012). By inserting sequences encoding a bioluminescent donor and a fluorescent acceptor protein into the primary sequence of G-protein coupled receptors (GPCR), we also developed a novel chimaeric BRET-based biosensor capable of detecting a volatile ligand with unprecedented sensitivity (Dacres et al., 2011). More recently, a maltose biosensor was constructed by genetically fusing a green fluorescent protein (GFP^2) at the N-terminus and a RLuc2 a genetic variant of Renilla luciferase (Loening et al., 2006) at the C-terminus of a maltose binding protein. The sensor responded to maltose and maltotriose and its applicability was demonstrated by measuring maltose concentrations in beer (Dacres et al., 2013). As these examples represent families of biosensors with potentially wide utility in biomedical and process control applications, we investigated whether it would be possible to develop a continuous flow real-time biosensor platform using a $BRET^2$ based PBP sensor. Whilst there are several examples of FRET-based microfluidic biosensors (Varghese et al., 2010), until recently there were very few, if any, cases where BRET-based biosensing had been demonstrated in the moving

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phase of a microfluidic device. Recently, however, we have combined microfluidics with BRET^H, using a microscope (Wu et al., 2014) and with BRET^2 and optical fibers (Le et al., 2014) to quantify thrombin activity.

Maltose, or malt sugar, is a small disaccharide found ubiquitously in beverages, cereal, pasta, and in many processed products which have been sweetened. Starch hydrolysis, whose main product is maltose, is an important step in brewing and food processing (Enevoldsen, 1978). During brewing, it is useful to measure maltose concentration to monitor the progress of the fermentation. HPLC (Sesta, 2006), gas chromatography (García Baños et al., 2000), electrospray mass spectroscopy (Mauri et al., 2002; Rozaklis et al., 2002) and enzyme-linked colorimetric or fluorescence detection kits (Edwards and Baeumner, 2013) are some of the commonly used analytical methods to measure maltose concentration accurately. However, these methods are generally time-consuming, require expensive reagents and instrumentation, and thus are not suitable for in-line monitoring for food and beverage processing applications. Accordingly, there is a need to develop alternative techniques that are quicker, cheaper and more importantly capable of measuring maltose concentration in a real-time, continuous flow format.

Here, we incorporate two innovations, the use of a luciferase with synthetically enhanced luminance of the maltose binding protein (MBP) sensor and liquid light guides, with microfluidic technology for greater photon transfer efficiency. In combination these improvements enabled extremely sensitive and continuous real-time quantification of maltose at the μL scale with a lag time of less than 100 s and EC_{50} of 1.3×10^{-7} M. To the best of our knowledge, continuous and real-time BRET detection of maltose on chip has not been previously reported. We believe that the system we demonstrate is the archetype for a family of simple and/or multichannel sensing devices that draw upon the full range of biological recognition elements available among the naturally assembled bacterial periplasmic binding proteins and engineered variants thereof. This device could lead to a generic transduction platform with a wide range of applications in areas including security, food and beverage quality control, process control for biotech industries as well as in environmental monitoring and health-care.

2. Device fabrication and experimental setup

Two-input microfluidic mixing devices with a serpentine common channel length of 18 mm, width of 200 μm, and height of 35 μm were fabricated from polydimethylsiloxane (PDMS) using standard photolithography. The geometry of the microfluidic chip was drawn using Adobe Illustrator CS4 and the pattern was printed on a transparency mask (5080 dpi, Allardice). Master patterns of the microfluidic devices were formed using a laminar dry film resist (Shipley 5038). Multiple layers of resist were laminated at 113 °C onto a substrate of polished stainless steel. The channels were lithographically patterned using a collimated UV source (=350–450 nm) operated at 20 mJ/cm^2 with a transparency film mask. After exposure, the test pattern was developed in 20% Na_2CO_3 solution. The pattern in the resist was subsequently replicated as a Nickel shim using an initial sputter deposition of 100 nm Ni followed by electroplating to a thickness of 150 μm. A 10/1 (w/w) ratio of PDMS and curing agent was poured over the shim and cured on a hotplate at 100 °C for 15 min. The height of the chip was controlled by a set of 1 mm high posts. The device was peeled off the shim and cut to the desired size. The reaction chamber (diameter 4 mm × height 1 mm) was formed by punching a hole at the end of the channel. It was sealed with a lid containing a top channel to allow escape of the reaction products. The PDMS chip was exposed to air plasma for two minutes and immediately bonded with a glass slide (Matsunami, 170 μm).

A dual syringe pump (Pump 11 Elite Programmable Syringe Pumps, Harvard Apparatus, Boston, USA) was used to introduce the reagents into two inlets through silicone tubing (0.5 mm ID, 1 mm OD, Gecko Optical, Australia). The BRET signals generated by the microfluidic chips were detected by the common butt of a bifurcated liquid light guide (Nikon) placed underneath the reaction chamber (Fig. 1). The butt has a core diameter of 6 mm and an NA of 0.59. The two branches of the light guide have core diameters of 4 mm and NA of 0.48. BRET emissions were recorded using two photo-multiplier tubes (PMTs) operating at gate time of 500 ms, one equipped with an RLuc/Clz400a emission filter (410 nm, bandpass 80 nm) and another with a GFP^2 emission filter (515 nm, bandpass 30 nm). Thanks to the use of a bifurcated light guide, it is not necessary to use a dichroic mirror to split the emission into two PMT tubes. This feature further simplifies the

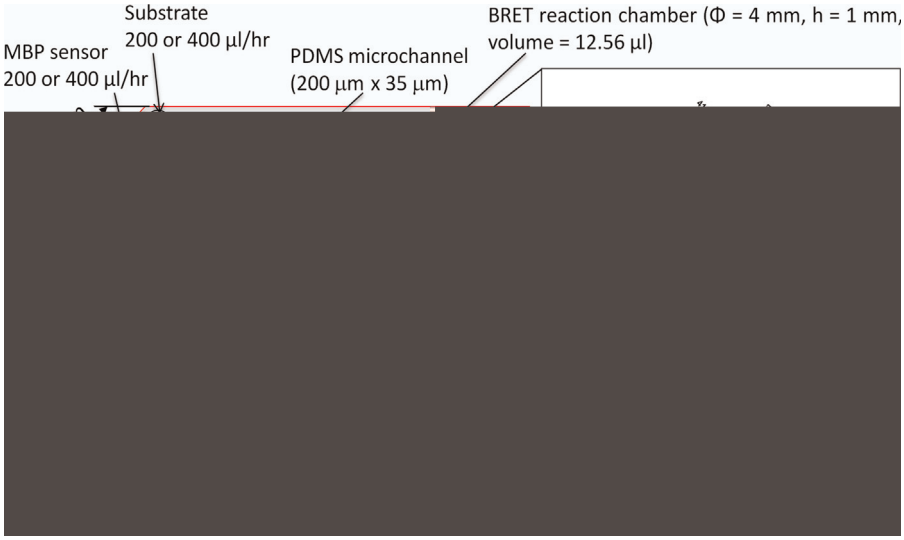

Fig. 1. Microfluidic BRET detection setup with a bifurcated liquid light guide, two band pass filters and two photomultiplier tubes (PMT). Inset cartoon shows the reaction mechanism of the MBP biosensor. Inset table shows the residence times for the serpentine section, the reaction chamber and the total device for flow rates of 200 μl/h and 400 μl/h.

4. Results and discussion

4.1. BRET signals and BRET^2 ratio

For the pre-incubated assays, BRET signals were detected approximately 10 s after initiating flow and stabilized completely after 130 s as shown in Fig. 2(A – incubated). We did a simulation by optical ray tracing software ZEMAX and collection efficiency was calculated to be 28.2%. Total luminance was detected at 12000–13000 counts/gate for Rluc and 3000–4500 counts/gate for GFP^2, respectively. Background noise was of the order of 300–400 counts/gate for Rluc and 100–200 counts/gate for GFP^2, respectively. The signal to background ratios were high: 32–40 for Rluc and 22–30 for GFP^2, respectively. The BRET^2 ratio traces (Fig. 2(B – incubated) were obtained by taking the ratios of the GFP^2 luminance over Rluc luminance for the control and positive assays, respectively. The negative control assay (0 μM) yielded a stable BRET^2 ratio of 0.25 and the positive assay containing 1 μM of maltose produced a BRET^2 ratio of 0.33: a 32% increase in BRET^2 ratio.

The typical BRET signals of the real-time assays (Fig. 2(C – real-time)) show similar characteristics to those of the pre-incubated assays, despite lower flow rates and lower luminescence. It is believed that the lower luminescence of the real-time assays is due to partial depletion of reagents prior to their reaching the reaction chambers (Le et al., 2014). Nonetheless, the typical BRET^2 ratios of the real-time assay (Fig. 2(D – real-time)) are also very similar to those of the pre-incubated assays. For the real-time assays, the negative control assay (0 μM) yielded a BRET^2 ratio of 0.27 while the positive assay produced a BRET^2 ratio of 0.34: a 26% increase in BRET^2 ratio.

As shown in Le et al. (2014), the mechanism of mixing of the reagents in our microfluidic chip is through diffusion. The BRET signal is strongly dependent on diffusion, i.e. better diffusion leads to a higher BRET signal due to better mixing of the MBP sensor and the substrate. Thus any change in the physical properties of the liquid samples such as change in viscosity affecting diffusion will also influence the BRET signals generated. A higher viscosity will result in less diffusion (Stokes-Einstein equation) which leads to reduction in BRET signals. Another factor that also leads to BRET signal reduction is bubble formation in the microchannel. Bubbles, especially in the reaction chamber, prevent the efficient mixing of the MBP sensor and the substrate.

4.2. Assays to detect maltose dissolved in DI water

In a previous paper, the BRET^2 tagged MBP biosensor was used to quantify maltose concentrations spanning three log units, ranging from to 1×10^{-8} M to 3.12×10^{-6} M with EC_{50} of 3.71×10^{-7} M (Dacres et al., 2013) (Fig. 3). We also showed that BRET is more suitable than FRET for measuring the change in distance induced by binding of maltose to MBP. In the current study, the log concentration response curve for on-chip detection of pre-incubated samples was effectively indistinguishable from that of the plate-based assay, having $EC_{50}=2.4\times10^{-7}$ M (with 95% confidence intervals from 9.95×10^{-8} M to 5.04×10^{-7} M). For the real-time assay, despite the lower flow rate, the luminance was 5000–7000 counts/gate for RLuc and 2000–3000 counts/gate for GFP^2, respectively. We obtained a similar sigmoidal dose response curve to that of the pre-incubated assay. EC_{50} of the real-time, on-chip assay was 1.3×10^{-7} M (with 95% confidence intervals from 1.01×10^{-7} M to 1.76×10^{-7} M).

The similarity of three analytical results (see Supporting information for the comparison of three assay formats) demonstrates that the MBP sensor responds consistently to maltose over a range of different assay formats and conditions. This is important because it shows that the microfluidic method, which is potentially much faster and cheaper, can replace more time-consuming methods such as plate-based assays. This is the first important step toward the implementation of this technique to real world applications. Moreover, the ability to continuously detect an analyte, such as maltose, using a simple microfluidic detection system simplifies the equipment required compared with FRET-based biosensors. Other advantages of microfluidic technology include fast response times, improved limits of detection, enhanced sensitivity, continuous operation and the potential for multiplexing in multiple channels. The method demonstrated here could be applied to a variety of applications such as point-of-care or field measurements.

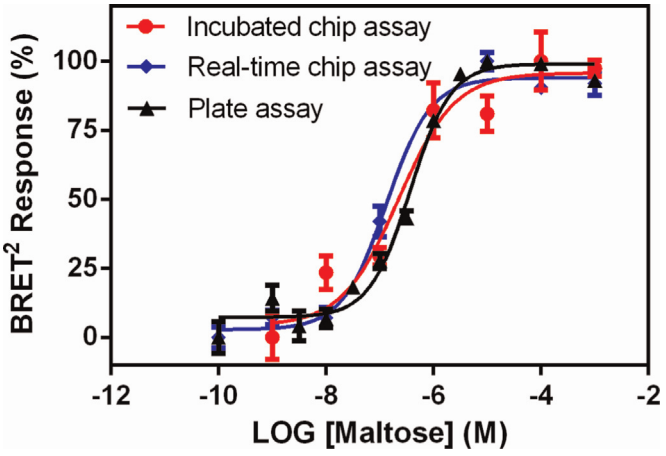

Fig. 3. Incubated and real-time on-chip dose response curves (mean ± SD, $n=3$) compared with incubated assay performed on 96-well plate (EC_{50} of 3.71×10^{-7} M (Dacres et al. 2013)). There are eight data points for the incubated and real-time chip assays. The incubated on-chip assay produced $EC_{50}=2.4\times10^{-7}$ M (with 95% confidence intervals from 9.95×10^{-8} M to 5.04×10^{-7} M) while the real-time on-chip assay produced $EC_{50}=1.3\times10^{-7}$ M (with 95% confidence intervals from 1.01×10^{-7} M to 1.76×10^{-7} M).

4.3. Real-time assay for detection of maltose in beer

Potential applications of BRET-based biosensors might include process control in the food and beverage industries, we selected a real-world example to test the feasibility of our method. Here, we performed real-time, on-chip measurements of maltose concentration in beer. The dilution curve (Fig. 4) was similar to those of pre-incubated and real-time assays of maltose in water. The calculated dilution fraction (ED_{50}) for maltose in beer was 6×10^{-5}. Using EC_{50} of 1.3×10^{-7} M for the real-time on-chip assay with maltose dissolved in water, an equivalent maltose concentration in beer was estimated to be 2.2 mM (with 95% confidence intervals from 1.52 mM to 3.22 mM). The commercial enzyme-linked colorimetric assay on the same beer sample yielded a concentration of maltose of 0.96 mM (with 95% confidence intervals from 0.36 mM to 1.56 mM) (see Supporting information for the experimental results). It is quite encouraging to see that the figure obtained by the microfluidic method is slightly larger than that obtained by the standard test kit and that there is some overlapping in the 95% confidence intervals of the two methods. To the best of our knowledge, this is the first time a BRET-based biosensor on a microfluidic device is capable of detection of maltose in beer, in real-time. It should be noted that EC_{50} of 1.3×10^{-7} M, which is used to calculate maltose concentration in beer, is obtained from different assays on a different chip to the assays with beer. Considering the lengthy time it takes to perform the enzyme-linked colorimetric assay, our microfluidic method could offer the answers almost immediately about

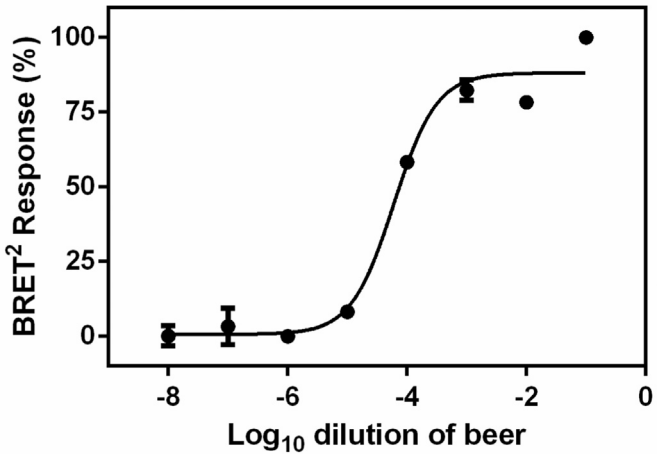

Fig. 4. Calibration curve (mean ± SD, $n=3$) for real time, on-chip detection of maltose in beer. The assay produced $EC_{50}=6.05\times10^{-5}$ dilution (with 95% confidence intervals from 4.16×10^{-5} M to 8.8×10^{-5} M). Using EC_{50} of 1.3×10^{-7} M for the real-time on-chip assay with maltose dissolved in water, the equivalent maltose concentration in beer was estimated to 2.2 mM (with 95% confidence intervals from 1.52 mM to 3.22 mM).

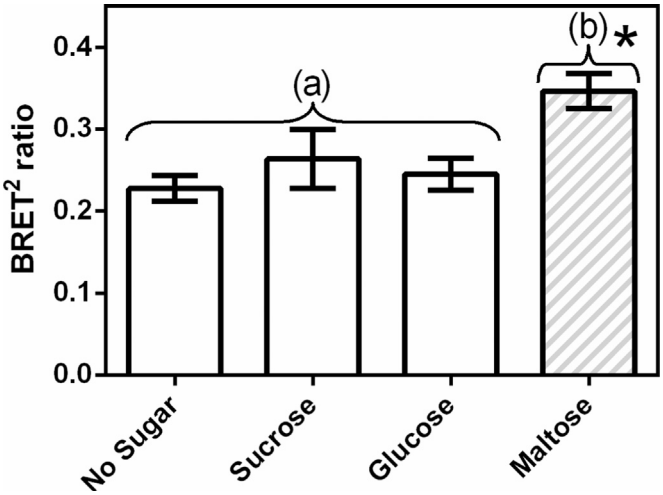

Fig. 5. Maltose specificity of the MBP sensor in a microfluidic format. Values are the mean ± SD of three independent experiments. Binding of 0.1 mM maltose yielded a 52% change in $BRET^2$ ratio. Reactions with glucose and sucrose resulted in 7% and 15% changes in the $BRET^2$ ratio, respectively. Only 0.1 mM maltose ($P=0.0007$) significantly changed the $BRET^2$ ratio (*$p<0.001$, 'a' is significantly different from 'b').

whether there is maltose in a complex sample and, in a few moments of data processing, what is the concentration of maltose in that sample. The technique proposed here could also be scaled up to multiplexing platforms with multiple channel devices to measure several samples simultaneously. This is a great advantage of the microfluidic approach for such applications as point-of-care testing and in-line monitoring.

4.4. Specificity assay

The specificity of the MBP BRET biosensor was investigated using microfluidic on-chip pre-incubated assays. In the absence of an analyte, the $BRET^2$ ratio for the control assay was 0.225 ± 0.016. Binding of maltose (Fig. 5) resulted in a large change in $BRET^2$ ratio with a mean $BRET^2$ ratio of 0.35 ± 0.021 for 0.1 mM maltose, an increase over the no sugar control of 52%. Reactions with glucose ($BRET^2$ ratio = 0.245 ± 0.019) and sucrose ($BRET^2$ ratio = 0.263 ± 0.036) resulted in 7% and 15% increases in $BRET^2$ ratio, respectively, confirming that the selectivity of the $BRET^2$-based sensor for maltose is maintained in a microfluidic format.

5. Conclusions

We have described a miniaturized, microfluidic system incorporating liquid light guides that accurately quantifies maltose over three log units using an enhanced $BRET^2$-based biosensor, which we believe is the first example of such combination. Thanks to the enhanced luminance, efficient light capture and continuous flow format, the system is capable of quantifying low levels of maltose in real-time. For maltose in DI water, the system showed similar log concentration-response curves to those obtained using a commercial 96-well plate instrument. In preliminary measurements in beer, the system estimated maltose concentration to be slightly lower than a standardized colorimetric assay albeit there was overlap of the 95% confidence intervals. Because results can be measured continuously and with a lag time of less than two minutes, this is a very a promising approach for in-line monitoring of a range of analytes in applications such as food and beverage processing and bioprocessing.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.06.032.

References

Dacres, H., Dumancic, M.M., Horne, I., Trowell, S.C., 2009a. Anal. Biochem. 385 (2), 194–202.
Dacres, H., Dumancic, M.M., Horne, I., Trowell, S.C., 2009b. Biosens. Bioelectron. 24 (5), 1164–1170.
Dacres, H., Michie, M., Anderson, A., Trowell, S.C., 2013. Biosens. Bioelectron. 41, 459–464.
Dacres, H., Wang, J., Dumancic, M.M., Trowell, S.C., 2009c. Anal. Chem. 82 (1), 432–435.
Dacres, H., Wang, J., Leitch, V., Horne, I., Anderson, A.R., Trowell, S.C., 2011. Biosens. Bioelectron. 29 (1), 119–124.
Dacres, Helen, Michie, Michelle, Trowell, Stephen C., 2012. Comparison of enhanced bioluminescence energy transfer donors for protease biosensors. Anal. Biochem. 424, 206–210.
Edwards, K.A., Baeumner, A.J., 2013. Anal. Chem. 85 (5), 2770–2778.
Enevoldsen, B.S., 1978. Denpun kagaku=J. Jpn. Soc. Starch Sci. 25 (2), 88–99.
Forster, T., 1959. Discuss. Faraday Soc. 27, 7–17.
García Baños, J.L., Olano, A., Corzo, N., 2000. Chromatographia 52 (3-4), 221–224.
Le, N.C.H., Gel, M., Zhu, Y., Glenn, F., Dacres, H., Wang, J., Anderson, A., Trowell, S.C., 2014. Biomicrofluidics (AIP), Submitted for publication.
Loening, A.M., Fenn, T.D., Wu, A.M., Gambhir, S.S., 2006. Protein Eng. Des. Sel. 19 (9), 391–400.
Mauri, P., Minoggio, M., Simonetti, P., Gardana, C., Pietta, P., 2002. Rapid Commun. Mass Spectrom. 16 (8), 743–748.
Pfleger, K.D.G., Eidne, K.A., 2006. Nat. Methods 3 (3), 165–174.
Rozaklis, T., Ramsay, S.L., Whitfield, P.D., Ranieri, E., Hopwood, J.J., Meikle, P.J., 2002. Clin. Chem. 48 (1), 131–139.
Sesta, G., 2006. Apidologie 37 (1), 84–90.
Varghese, S.S., Zhu, Y., Davis, T.J., Trowell, S.C., 2010. Lab Chip 10 (11), 1355–1364.
Wu, N., Dacres, H., Anderson, A., Trowell, S.C., Zhu, Y., 2014. Comparison of static and microfluidic protease assays using modified bioluminescence resonance energy transfer chemistry. PLoS ONE 9 (2), e88399, http://dx.doi.org/http://dx.doi.org/10.1371/journal.pone.0088399.