Published in final edited form as:

Curr Protoc Cytom. 2010 October ; 0 13: Unit-13.1217. doi:10.1002/0471142956.cy1312s54.

MICROSPHERE-BASED FLOW CYTOMETRY PROTEASE ASSAYS FOR USE IN PROTEASE ACTIVITY DETECTION AND HIGH-THROUGHPUT SCREENING

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Abstract

This protocol describes microsphere-based protease assays for use in flow cytometry and high-throughput screening. This platform measures a loss of fluorescence from the surface of a microsphere due to the cleavage of an attached fluorescent protease substrate by a suitable protease enzyme. The assay format can be adapted to any site or protein specific protease of interest and results can be measured in both real time and as end point fluorescence assays on a flow cytometer. End point assays are easily adapted to microplate format for flow cytometry high-throughput analysis and inhibitor screening.

INTRODUCTION

Proteases, proteins that break down other proteins through the hydrolysis of peptide bonds, are a large and diverse group (Turk, 2006). The protease family of proteins is considered to be one of the major targets for small molecule drug discovery (Cudic and Fields, 2009). Initially, proteases were primarily thought of as protein degrading enzymes; however, it is now clear that proteolytic mechanisms are highly regulated components of cellular signaling pathways. The improper regulation of specific human proteases involved in cellular signaling can lead to human diseases including inflammatory disease, thrombosis, osteoporosis, cardiovascular and neurological disorders, and increased growth and metastasis in specific cancers (Turk, 2006). Proteases of clinical significance include human proteases which are improperly regulated (Turk, 2006), bacterial pathogenic proteases that mediate the effects of toxins (Barth, et al., 2004), and viral proteases that process viral precursor proteins controlling viral life cycles (Steuber and Hilgenfeld, 2010).

Many protease assays are based on fluorescence resonance energy transfer (FRET), where a short peptide containing a protease cleavage site is placed between two fluorophores which are FRET pairs. The small peptides typically used in FRET assays fail to address protease/substrate recognition elements distal from the protease cleavage site, which in many cases account for protease/substrate specificity (Vitale, et al., 2000; Chopra, et al., 2003; Rossetto,

et al., 1994 ; Breidenbach and Brunger, 2004). These distal elements may also be areas of interest for pharmacological inhibition of specific proteases (Eubanks, et al., 2007; Silhar, et al., 2010). Using microsphere-based flow cytometry assays it is possible to use full-length protease substrates in a protease assay (Saunders, et al., 2010), which may allow detection of inhibitors of protease/substrate interactions distal from the protease active site or substrate cleavage site. Flow cytometry also enables the use of multiplex microsphere sets to assay several proteases in the same assay volume. Adaptation of a flow cytometry assay to high-throughput screening applications makes a cost effective and robust platform for drug screening against target proteases (Saunders, et al., 2010). This unit describes the basic protocol of microsphere-based flow cytometry protease assays from substrate preparation, attachment to microspheres, data collection, and adaptation to high-throughput screening.

STRATEGIC PLANNING

Substrate preparation

Recombinant fluorescent proteins that serve as protease substrates in these studies have a biotinylated lysine residue at one end and a green fluorescent protein (GFP) at the other end. Only in this configuration will a loss of fluorescence be detected from the surface of the microsphere upon proteolytic cleavage (Figure 13.12.1). Sub-cloning, expression and purification from *E. coli* is our preferred method of obtaining protease substrates, using protein attachment tags at one end and a fluorescent protein expressed on the other end of protease substrates. In the case of proteins not capable of being expressed in *E. coli*, other approaches such as mammalian or insect cell expression followed by protein purification is a valid method as well. In this case, it should be noted that the substrate will not be biotinylated *in vivo* during expression if biotin-avidin attachment chemistry is being used. Only *E. coli* will biotinylate *in vitro* using either the Promega PinPoint™ system or other biotinylation sequences and bacterial strains from Avidity LLC and Life Technologies, Inc. A short amino acid sequence can be used to biotinylate proteins expressed from other systems *in vitro* after protein purification using the BirA biotin ligase enzyme available from Avidity LLC.

Choice of attachment chemistry is also an important consideration, as high affinity binding pairs will use less substrate to label the microsphere efficiently and will stay bound on the same microsphere for longer periods of time. Most work on these assays to date uses biotin-avidin attachment by virtue of a biotinylation tag expressed on the N-terminus of the protease substrate (Saunders, et al., 2010; Saunders, et al., 2006). Much of this work has been done by modifying the Promega PinPoint™ protein expression plasmid, by sub-cloning GFP C-terminal to the multiple cloning site in this plasmid. Bacterial strains such as BL21 (DE3) pLys S efficiently biotinylate this amino acid sequence specifically near the N-terminus when expressed and specific biotinylation can be increased using the AVB 101 bacterial strain available from Avidity LLC. Purification of biotinylated protease substrates from bacterial cell extracts can be done on SoftLink™ streptavidin resin available from Promega. A brief description of a typical protein purification is described in Support Protocol 1. Furthermore, biotinylated substrates are easily attached to avidin or streptavidin coated microspheres (Saunders, et al., 2006). While it is relatively straightforward to

covalently couple streptavidin to carboxyl functionalized microspheres (Bangs Laboratories, TechNote 205), it is also possible to purchase streptavidin coated microspheres at high concentrations from multiple commercial sources.

Other potential methods of substrate to microsphere attachment include using polyhistidine tags for both protein purification and attachment to Ni^{2+} coated microspheres (Lauer and Nolan, 2002), or GST fusion protein attachment to microspheres containing reduced glutathione (Tessema, et al., 2006). Due to the nature of this assay, which measures loss of fluorescence, avidin/streptavidin microspheres with biotinylated substrates are recommended over other attachment chemistry due to their tight binding with little or no dissociation from microspheres over periods of days, as will be highlighted in the protocols here.

One important consideration when using biotin-avidin attachment is to remove all free biotin from the protein preparation. Softlink streptavidin is eluted using 5 mM biotin, which will effectively block streptavidin or avidin microspheres from binding biotinylated protein. Protein purifications must be filtered or dialyzed to reduce the amount of biotin as much as possible (at least to femtomolar levels) to prevent free biotin binding to avidin or streptavidin from blocking biotinylated protein binding. Typical dialysis conditions to effectively remove biotin are 1 to 12,000 volumes, or 1 ml protein sample dialyzed against 12 L of biotin free buffer. Serial dialysis against 3 L of buffer at each stage improves the result.

Choice of Microspheres

Most commercially available flow cytometers are capable of easily detecting microsphere sizes between 1 μm and 30 μm diameter in forward and side scatter bivariate plots. It is possible to conjugate streptavidin to microspheres using established protocols, or to purchase microspheres with attachment chemistry already conjugated at high levels from multiple sources (e.g., Spherotech, Bangs Labs, Luminex). Microsphere choice may depend on costs of the microspheres or ease of preparation. If multiple protease assays will be performed in the same reaction volume via multiplex microsphere sets, an appropriate multiplex microsphere set must be chosen for conjugation with attachment molecules or purchased with attachment molecules present. Multiplex microsphere sets consist of microspheres bearing different intensities of fluorescence in a specific fluorescence channel, which can easily be distinguished in a histogram plot of fluorescence intensity. If multiplex microsphere sets are being used they should fluoresce in a different channel than that of the protease substrate. Streptavidin coated multiplex microspheres purchased from Spherotech Inc. (SVFA-2558-6K) perform well and are used in the assays described in this unit. Other sources or attachment chemistries are available and should be appropriate for the application and protein purification strategy. All protocols here describe biotin/streptavidin chemistry.

BASIC PROTOCOL: MICROSPHERE-BASED PROTEASE ASSAYS BY FLOW CYTOMETRY

This protocol describes the binding of fluorescent protease substrates to microspheres, washing of the microspheres to remove all protease substrate still in solution, as well as real-time and end-point fluorescence measurements by flow cytometry for specific protease cleavage.

Materials

Protease buffer (50 mM HEPES, 100 mM NaCl, 1 mg/ml Bovine Serum Albumin and 0.025% Tween-20 pH 7.4).

Purified and dialyzed biotinylated fluorescent protease substrate (see Support protocol 1)

Purified or purchased protease of interest

Microspheres functionalized with streptavidin or avidin at a stock concentration between 10^6 and 10^9 /ml (Spherotech Inc.)

Microcentrifuge tubes (1.5 ml)

A mixing device such as a rotator or Nutator[®]

A microcentrifuge capable of spinning microcentrifuge tubes between 13,000 and $16,000 \times g$

A flow cytometer capable of measuring EGFP fluorescence (for example, with excitation at 488 nm and measuring 505–550nm fluorescence)

NOTE: If multiplex microsphere sets are used the cytometer must be able to detect the fluorescence channel of microsphere fluorescence as well as substrate fluorescence.

Bind fluorescent protease substrates to microspheres

- 1 Place protease buffer, approximately 10^5 to 10^6 microspheres and the appropriate concentration of protease substrate for near microsphere saturation (as determined by protein titration in Support Protocol 2) into a microcentrifuge tube, in 500 μ L total volume. If multiplex microsphere sets are being used set up in separate microcentrifuge tubes for each set.

Once specific protease substrate binding to microspheres has been demonstrated as in Support Protocol 2, a concentration of substrate which results in a high degree of specific binding to microspheres must be chosen for use in protease assays. Typically, this amount will be near the saturation point of the microsphere with a low level of non-specific binding observed in the sample with excess biotin at the same concentration. In the sample protein to microsphere titrations shown in Figure 13.12.2, a concentration ~ 100 nM biotinylated protein would be used for ppGFP and 300 nM for SNAP-25 GFP to achieve the

desired level of fluorescence. Increasing the amount of microspheres used in binding substrates by up to two orders of magnitude does not appear to have much effect on mean/median fluorescence values observed on the microspheres, most likely due to the low number of binding sites on microspheres compared to the protease substrate in solution.

- 2 Incubate the mixture in a mixing device such as a rotator or neutator for 1 hour covered from light.
- 3 Centrifuge microspheres at $13,000$ to $16,000 \times g$ for 1–2 minutes, a small microsphere pellet should be visible at the bottom of the microcentrifuge tube when this is done.
- 4 Carefully remove all buffer from the tube while leaving the microsphere pellet intact.
- 5 Add 500 μ l buffer to the microsphere pellet and vortex to wash the pellet.
- 6 Repeat steps 3–5 twice for a total of three wash steps.

Microspheres must be bound with substrate and washed to remove all non-bound substrate prior to addition of protease. Concentrations of 0.025% Tween-20 in the buffer help to stabilize microspheres during the wash steps due to increased microsphere pelleting during centrifugation steps required for removal of soluble substrate after binding. For the experiments described here, a protease buffer optimized for the B. anthracis lethal factor and C. botulinum light chain proteases, consisting of 50 mM HEPES, 100 mM NaCl, 1 mg/ml BSA, and 0.025% Tween-20 pH 7.4, was used and is referred to as protease buffer. Protease buffer will need to be optimized for individual proteases.

- 7 If multiplex microsphere sets with multiple substrates are being used, combine all of the microspheres into one tube after the wash steps. This will require suspending each microsphere set in a smaller amount of buffer (100 μ l) and combining them, then adding protease buffer to reach a 500 μ l volume.

Multiple protease substrates or proteases can be analyzed at once through the use of multiplex microsphere sets performed in the same protocol if the buffers are compatible. Biotinylated protein bound to streptavidin multiplex microspheres is advised for multiplex assays as no dissociation has been observed from streptavidin microspheres over several hours.

Analyze binding to microspheres

- 8 Remove 25 μ l of the microspheres; add them to 475 μ l buffer and analyze with a flow cytometer. Gate microspheres based upon a bivariate plot of forward

scatter vs. size scatter; take care to gate only single microspheres and not microsphere aggregates (Figure 13.12.3A).

Perform protease assay

- 9 Aliquot microspheres into separate sample tubes for protease assays; the number of tubes depends on the number of assays desired and the number of microspheres bound. The total volume of the sample will depend on how long the desired assay will take, and how many microspheres per microliter are present and will be brought up to appropriate volumes using protease buffer.

Typical sample sizes are 500 μ L total volume, but can vary from 100 μ L to several ml. For example if ten 500 μ L samples will be set up from a starting volume of 500 μ L microspheres, each sample will contain 50 μ L microspheres and 450 μ L protease buffer.

- 10 Display a histogram of fluorescence in the proper fluorescence channel (e.g. FL1 for GFP or FITC, FL2 for PE etc.) for your substrate and gate on the microsphere size gate (Figure 13.12.3B). If multiplex microspheres are used make a histogram of the fluorescence channel of the microspheres and gate each microsphere population on the histogram (Figure 13.12.3C). If there is overlap between substrate fluorescence and microsphere fluorescence, compensation will need to be used to determine and gate the microsphere populations. If only one protease substrate is being used this step is not required.

The multiplex microspheres used in figure 13.12.3C each have a small amount of their population, which appears as a small secondary peak of higher fluorescence intensity and can lead to a small amount of overlap into additional microsphere populations. This phenomenon is common with some multiplex microspheres but can be gated out on the histogram plot. Small secondary shoulders which overlap with another microsphere population will lead to approximately 2–5 % overlap of one microsphere population into another.

- 11 Make a bivariate plot of substrate fluorescence vs. time (Figure 13.12.3D). Set gating of this plot to display and record only size gated microspheres in the gate from step 9 (Figure 13.12.3A). If multiplex microspheres and several substrates are being used, set up plots based upon gates drawn on histogram plots in step 10 (Figure 13.12.3C).

The length of time recorded will depend on the volume of the sample and the flow rate of the cytometer. Most commercial cytometers will acquire samples of 500 μ L for 20 to 30 minutes on a slow flow rate.

- 12 Run a sample of microspheres with no protease added for 60 seconds to get an average median starting value, remove the tube from the flow cytometer while it is still running and add protease to the sample, vortex or mix quickly, then put the tube back on the flow cytometer.

Protease concentration must be determined empirically for maximum or desired amount of cleavage. It is suggested that for the first experiments with a protease a concentration range of protease be tested on multiple samples of substrate-bound microspheres. Substrate cleavage will be protease concentration dependent as shown in the example using Botulinum Neurotoxin type A Light Chain with the substrate biotinylated SNAP-25 GFP (Figure 13.12.4).

- 13** Measure median/mean fluorescence values over time to measure loss of fluorescence from the microsphere due to proteolytic cleavage.

Real-time protease cleavage measurements will demonstrate proteolytic cleavage of fluorescent substrates over a relatively short period of time (30 minutes). End-point assays can also be performed at chosen time points instead of continuous real-time measurements. Start at step 10, and prepare samples with and without protease; incubate at the desired temperature on a mixing device and analyze using the mean or median fluorescence values after the desired incubation time, measured on a histogram plot of substrate fluorescence (Figure 13.12.3B). Protease concentrations can be adjusted to give optimal or desired substrate cleavage rates either in real-time or as end-point assays. If multiple substrates with multiple proteases are used, all of the proteases can be added together in a mixture with fluorescence values of each substrate monitored in real-time by gating on microsphere histogram plots. Separate substrate analysis can be done in flow cytometry analysis software such as FlowJo, FCS Express, or in our case we used a custom program known as IDL Hyperview.

SUPPORT PROTOCOL 1: GROWTH AND PURIFICATION OF BIOTINYLATED PROTEINS FROM BACTERIA

This protocol describes the steps to transform and growth bacteria, as well as to purify the tagged protein.

Materials

Plasmid containing a biotinylation sequence on one end (Pinpoint from Promega, or AviTag vectors from Avidity LLC., Gateway plasmids from Life Technologies) and a fluorescent protein on the other end (e.g. GFP, YFP, etc.), cloned in-frame with protease substrate sequence in between.

Calcium or electro-competent expression bacteria which will biotinylate protein sequences *in vivo*.

NOTE: biotinylated protein purifications have been done using BL21 (DE3) pLys S bacteria available from Promega. Additional specific biotinylation can be achieved by using the AVB 101 strain available from Avidity LLC.

Terrific broth (Fisher Scientific)

Appropriate resistance antibiotic for plasmid selection (Carbenicillin or Ampicillin for Pinpoint plasmids and chloramphenicol for the strains used here)

LB agar plates with 50 µg/ml carbenicillin/ampicillin and 34 µg/ml chloramphenicol

Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich)

Centrifuge capable of forces up to 25,000 × g

Centrifuge tubes and rotors to sediment 200–500 ml culture and 30–50 ml bacterial lysate

Column chromatography system or peristaltic pump with glass column

Phosphate buffered saline pH 7.2 (PBS)

Spectrophotometer capable of measuring absorbance values at a wavelength of 600 nm

Softlink streptavidin resin (Promega)

Biotin (Sigma-Aldrich)

Ultra centrifugal filter units, Mw cutoff (Millipore)

12 L protease buffer for dialysis of purified substrates (For *B. anthracis* lethal factor and *C. botulinum* light chain proteases: 50 mM HEPES, 100 mM NaCl pH 7.4, see recipe).

Dialysis membrane with appropriate Mw cutoff values

Dialysis clamps

Transform bacteria

- 1 Clone and sequence plasmid containing the biotinylation tag, protease substrate and protein fluorophore by preferred method.
- 2 Transform plasmid into *E.coli* expression strain by preferred method (Seidman 1997). Plate on LB agar plates with appropriate antibiotic resistance (50 µg/ml ampicillin or carbenicillin for pinpoint vectors, 34 µg/ml chloramphenicol for pLysS or AVB 101 strains).
- 3 Grow colonies on plates overnight at 37°C.
- 4 Pick colonies from plates and inoculate 3 ml Terrific broth (TB) cultures with appropriate antibiotics. Grow overnight at 37° C.
- 5 Pour 3 ml cultures into 200 to 500 ml of TB with appropriate antibiotics and grow at 37° C for 2–4 hours checking light absorbance at 600 nm (A600) every 30 minutes.
- 6 When bacterial cells reach an optical density (A600) between 0.6 and 0.8, induce by adding IPTG to a final concentration of 100 µM.
- 7 Grow at 30° C for 12–15 hours (overnight)

- 8 Spin down bacteria at $5,000 \times g$ for 10 minutes in a centrifuge with appropriate centrifuge tubes and rotor. Remove media from cell pellet. GFP expressing cells should have a visible green tint at this point if high expression levels have been reached.
- 9 Suspend bacterial cell pellet in 30 to 50 ml PBS.

Concentrate proteins

- 10 Lyse cells using freeze/thaw, chemical methods, sonication or preferred method.
- 11 Spin down lysate at $25,000$ to $35,000 \times g$ for 30 minutes. This should clarify the lysate and remove all membrane and lipid components. GFP expressing bacterial lysate should be visibly green. Keep clarified lysate for loading onto column.
- 12 Pack a glass column or appropriate column for a protein purification system with 5 ml Softlink streptavidin resin and equilibrate with PBS. Load lysate flowing at a rate of 1 ml per minute and collect flow through.
- 13 Wash resin with 100 ml PBS to flow through all unbound protein.
- 14 Load 5 ml of 5 mM biotin in PBS onto column with Softlink streptavidin resin. Stop flow and incubate for at least 1 hour.
- 15 Elute column with 5 mM biotin in PBS. Collect elution until no UV absorption at 280 nm is detected, or collect ~ 30 ml if no detection device is being used.
- 16 Concentrate elution by spinning at $5,000 \times g$ for 10 minutes in a molecular weight (Mw) cutoff filter of appropriate size (e.g. a molecular weight smaller than your protein.) Spin the maximum elution volume in the filter unit, discard flow through, add more elution and re-spin until the entire eluted sample is concentrated into a 1–5 ml volume. GFP proteins should be visibly green.

Purify proteins

- 17 Add concentrated protein into a dialysis filter pre-wet in the same buffer as for the protease assay and securely fasten clamps. Dialyze at least four hours. It is recommended to use multiple rounds of dialysis to remove free biotin. This requires 12 L of buffer to effectively remove biotin from 1–5 ml in 5 mM biotin used in the protein elution.

This step is critical in ensuring biotinylated protein binding to avidin or streptavidin microspheres.

- 18 Determine protein concentration using an absorbance measurement and the extinction coefficient of the protein at 280 nm. Other preferred methods such as Bradford assays can be used as well.
- 19 Perform a titration of biotinylated fluorescent protein to streptavidin or avidin microspheres (see Support protocol 2).

SUPPORT PROTOCOL 2: SPECIFIC MICROSPHERE BINDING MEASUREMENTS FOR OPTIMAL DISPLAY OF PROTEASE SUBSTRATES

This protocol describes a basic protein titration onto the surface of a microsphere via biotinylated protein binding to streptavidin microspheres. Samples are set up with biotinylated protease substrate alone with streptavidin coated microspheres or a pre-block biotin treatment to microspheres prior to substrate addition. The biotin block samples will measure non-specific binding to microspheres which can be subtracted from the total amount of bound substrate to measure specific binding.

Materials

Streptavidin or avidin coated microspheres, either prepared in lab or commercially purchased (Spherotech Inc.)

Phosphate buffered saline pH 7.2 (PBS)

Purified protease substrate biotinylated on one terminus with a fluorophore for detection on the other terminus (Support protocol 1)

Microcentrifuge tubes (1.5 ml)

Mixing device such as a rotator or neutator

A flow cytometer capable of excitation and detection at appropriate wavelength for substrate fluorophore (488 nm excitation for GFP, detection in 505–550 nm range)

1. Determine the appropriate concentration range for the protein titration and number of samples desired. This should cover approximately two orders of magnitude spanning from 1 nM to 500 nM of protein (Figure 13.12.2). It is recommended to set up between six and eight 500 μ l samples in this concentration range. Approximately 10^4 to 10^5 microspheres per ml will be needed in each sample. Calculate the volume of buffer (PBS), protein and microspheres needed in a 500 μ l volume of the desired protein concentration for each sample. Samples will be set up in duplicate, one sample pre-blocked with biotin and one unblocked sample.
2. Add the appropriate volume of PBS calculated above to each sample tube.
3. Add the appropriate volume of microspheres to each tube to yield between 10^4 and 10^5 microspheres/ ml.
4. Add an excess of biotin to the blocked set of samples containing the buffer and microspheres, 1 μ M to 5 μ M biotin is sufficient to block specific binding. Incubate both blocked and unblocked microspheres at room temperature on a mixing device for 30 minutes.
5. Add biotinylated, fluorescent protease substrate at the correct concentration to all samples, both blocked and unblocked.
6. Incubate at room temperature for 1 hour on a mixing device covered from light.

7. Run samples on a flow cytometer collecting 10,000 size gated events per sample. Microspheres should be gated on size based on a bivariate plot of forward and size scatter determined empirically by the microsphere types used (Figure 13.12.3A). Set up a green fluorescence histogram plot to measure mean or median green fluorescence for each sample (Figure 13.12.3B). If using a cytometer with adjustable fluorescence PMT settings, the PMT settings should be determined by testing first the highest concentration of unblocked fluorescent protease substrate bound microsphere. Adjust the PMT setting in the fluorescence channel so the mean fluorescence readings from size gated samples are not off-scale on a histogram plot of fluorescence (log scale), but are very high (90% of the maximum channel number is a recommended value).
8. Plot unblocked and blocked fluorescence mean/median values vs. concentration of fluorescent protease substrate (Figure 13.12.2). Subtract median/mean blocked values from median/mean unblocked values and plot these values as specific binding.

The use of this protocol will yield a binding curve of the protease substrate to the microspheres and define specific vs. non-specific binding for each concentration of protease substrate in median or mean fluorescence units (Figure 13.12.2). Ideally, non-specific binding determined by the fluorescence level of the blocked samples, will be relatively low compared to specific binding. High non-specific binding could be due to hydrophobic protein/peptide interactions with microspheres. If the fluorophore is measured in a fluorescence channel with standard microsphere sets available (e.g., FITC, or phycoerythrin), a standard curve can be made to estimate the number of molecules on the surface of the microsphere in MESF values. Two typical biotinylated fluorescent protein titrations to streptavidin microspheres are shown in Figure 13.12.2, one with little non-specific binding (Figure 13.12.2A) and one with relatively high non-specific binding (Figure 13.12.2B).

SUPPORT PROTOCOL 3: ADAPTATION OF MICROSPHERE-BASED PROTEASE ASSAYS TO HIGH-THROUGHPUT SCREENING BY FLOW CYTOMETRY

The microsphere-based protease assays described above can be adapted to high-throughput screening applications by performing assays in 96 or 384 well microplates and using equipment to run these plates on commercial flow cytometry systems. The system used in these studies is the HyperCyt high-throughput flow cytometry system available from Intellicyt (Albuquerque, NM). Other plate-based sample acquisition devices such as the Beckton Dickinson (BD) LSR II HTS System or other flow cytometry HTS plate samplers would be equally appropriate for these applications. It should be noted that an HTS flow cytometry system is required here, as a typical fluorescence plate reader will be incapable of determining loss of fluorescence from the surface of microspheres and instead evaluates total liquid fluorescence in microplate wells.

Materials

Streptavidin or avidin coated microspheres at a stock concentration of 10^6 to 10^9 per ml

Purified or purchased protease of interest

Purified fluorescent protease substrate with biotin at one end and a fluorophore at the other end with the protease substrate or cleavage site in between (Support protocol 1)

Protease buffer

Microcentrifuge tubes (1.5 ml)

Centrifuge capable of spinning 1.5 ml tubes between 13,000 and $16,000 \times g$

96 or 384 well microplates

Test compounds (chemical libraries etc.)

A flow cytometer with appropriate excitation lasers and emissions filters for excitation and detection of fluorophore incorporated in protease substrate.

Plate acquisition device for flow cytometry (e.g., HyperCyt, BD LSR II HTS, or other)

Bind substrate to microspheres

- 1 Bind biotinylated substrate to streptavidin or avidin microsphere for 1 hour as described in the Basic Protocol. For multiplex microspheres and several substrates, bind each microsphere/substrate population separately.

When setting up the assay, the appropriate number of beads must be used. Typical well volumes are 10–25 μ l per well for 96 well plates and 6–15 μ l volumes per well for 384 well plates. It is desirable to input approximately 1,000 to 5,000 microspheres per microplate well if using the Hypercyt flow cytometry system, which aspirates approximately 2 μ l per well in 1 second. HTS cytometry plate systems which use the entire well volume during analysis may lower total required microsphere numbers than systems such as Hypercyt, which analyzes only 2 μ l of the total volume. Calculations of initial microsphere numbers should take into account ~30% to 50% of microsphere loss during sample preparation.

- 2 Spin down substrate bound microspheres at 13,000 to $16,000 \times g$ for 1–2 minutes, remove buffer and re-suspend in 500 μ l protease buffer. Repeat twice.
- 3 If multiple microspheres/substrates are being used combine into one sample.

Perform protease assay

- 4 Remove a small volume (10–25 μ l) and suspend in 500 μ l of protease buffer. Acquire on a flow cytometer and draw size-based and fluorescence gates as described in the Basic Protocol.
- 5 Add appropriate amount of protease buffer to microspheres to fill desired amount of 96 or 384 well microplates with desired volume of microspheres.

- 6 Using multi-channel pipettes or robotic systems, add microspheres suspended in buffer to 96 well or 384 well plates. The volume of microspheres added depends upon the desired final volume of the assay and the type of microplate used (e.g. 96 or 384 well plates).
- 7 Add test compounds (e.g. concentrated chemical library compounds) to microplate wells containing microspheres and buffer in the 96 or 384 well plates. Do not add test compounds to positive and negative control wells.
- 8 Add protease to test wells and negative control wells.

The optimal protease concentration for these assays should be determined empirically with Basic Protocol using end point assays. The volume of protease depends upon the final concentration desired. If multiple proteases are being assayed against multiple substrates, they can be added into the plate together. Do not add protease to positive control wells as these wells mimic complete inhibition. Positive and negative control wells without protease or test compound, respectively, must also be set up and measured to calculate a Z' Factor for the assay.

- 9 Cover plate and incubate on a rotating device for 1 to 2 hours. This should be determined empirically depending on end-point assays described in the Basic Protocol.

Microplate high-throughput flow cytometry protease assays are performed as end-point assays with a typical 1–2 hour incubation of protease in the presence of substrate bearing microspheres and test compounds.

Collect and analyze data

- 10 Acquire microplate on high-throughput flow cytometry device and collect data on flow cytometer.
- 11 Analyze data using data analysis software and calculate median fluorescence values for fluorescent substrates on microspheres in each well. If multiple microspheres/substrates are being analyzed calculate median fluorescence values for each microsphere/substrate.
- 12 Calculate an average median fluorescence for positive and negative control wells along with standard deviation in median fluorescence between control wells.
- 13 Calculate a Z' factor for the assay using the formula below, where Z' is a statistic for assessing assay quality (Zhang, et al., 1999). If multiple substrates/microspheres are being used calculate Z' separately for each microsphere/substrate type.

$$Z' = 1 - \frac{3(\text{StdevPositivecontrol} + \text{StdevNegativecontrol})}{\text{AvPositivecontrol} - \text{AvNegativecontrol}} \quad [\text{Equation 13.12.1}]$$

Stdev is the standard deviation of the median fluorescence of the positive control well and negative control well, and Av is the averaged median fluorescence values for positive and negative control wells. A Z' of 0.5 or better is considered indicative of a high quality assay suitable for HTS (Zhang, et al., 1999).

- 14 Convert compound well values into percent inhibition using the following formula:

$$\% \text{ inhibition} = 100 * \frac{\text{Value} - \text{AvNegativecontrol}}{\text{AvPositivecontrol} - \text{AvNegativecontrol}} \quad [\text{Equation 13.12.2}]$$

Value is the median fluorescence value for the test well with compound, and AvPositive control and AvNegative control are the averaged median fluorescence values for positive control and negative control wells.

- 15 Determine compounds with percent inhibition values above 30%. Perform dose-response assays using increasing amount of compound in microsphere based assays over two orders of magnitude, higher and lower than that screened to get an IC₅₀ value of the compound toward the protease of interest.

COMMENTARY

Background information

Proteases are currently one of the major families of proteins under investigation as potential drug targets. There is currently no overlying methodology to investigate protease activity *in vitro* with applications to high-throughput screening for protease inhibitors. Most protease assays use fluorescence resonance energy transfer (FRET) methods placing protease cleavage sites between two fluorophores capable of FRET. This methodology does not take into account protease/substrate recognition elements distal to the protease cleavage site, which accounts for much of the specificity of some proteases. Using flow cytometry and multiplex microsphere sets it is possible to use full-length protease substrates in assays with multiple proteases or multiple substrates at once in high-throughput screening. These assays can be adapted to any site specific or protein specific protease of interest for assaying protease activity over time and for use in flow cytometry based high-throughput screening for protease inhibitors.

Critical steps and Troubleshooting

Binding of fluorescent protease substrates to microspheres—Choice of attachment chemistry for substrates to microspheres may affect levels of fluorescent protease substrates on microspheres. In the case of avidin/streptavidin microspheres with biotinylated proteins, great care must be taken to remove all free biotin from protease substrates. Free biotin will bind to avidin/streptavidin and prevent biotinylated substrate from binding. Purification of biotinylated protein from Softlink streptavidin is done using 5 mM biotin. Effective removal of biotin can be accomplished by dialyzing 1–5 ml of purified concentrated substrate in 5 mM biotin against 3 L of buffer, four times (12 L total) for four hours each time. This will remove enough free biotin from the protein preparation to prevent

biotin from binding to avidin/streptavidin on the microsphere and blocking biotinylated protein binding.

GST fusion tags and 6X Histidine tags bound to reduced glutathione or Ni^{2+} microspheres, respectively, may require higher concentration of protein than biotinylated protein binding to avidin/streptavidin microspheres. If low fluorescence values are seen during initial protein titrations then concentrations may be increased to achieve efficient binding. It should also be noted that Ni or glutathione functionalized microspheres may allow substrate dissociation over time or prolonged storage due to the relative K_D of Ni to 6X Histidine and GST to reduced glutathione compared to biotinylated protein/avidin. Biotinylated protein or peptide bound to avidin/streptavidin microspheres are stably bound for several days of storage at 4°C.

Lack of proteolytic cleavage of substrates off of microspheres—In cases where protease substrates are bound to microspheres but the proteolytic cleavage is not detected, higher protease concentrations may be used. With insufficient wash steps, soluble substrate remains in solution and may be cleaved instead of substrate bound to microspheres, resulting in a substrate sink. Protease activity can be measured by cleavage of substrates in solution, followed by Western blot with antibodies to the protease substrate, or using streptavidin HRP or AP blots to biotinylated proteins, anti-His antibodies to His tagged proteins, etc. Size differences by Western blot analysis will indicate protease cleavage. In the case of protease purified in the laboratory, activity will need to be verified by either microsphere-based assays or secondary assays such as FRET based assays. Protease purification methods will vary for different proteases and it is suggested to use established protocols when available. Dialysis of protease into protease buffer after purification is also necessary to remove high salt, imidazole or other compounds used during purification, which may lead to a lack of proteolytic activity.

Current literature should be taken into consideration when short cleavage site substrates are being used instead of full-length protease substrates. In some cases, protease cleavage sites alone are not sufficient for cleavage by their protease as they lack required distal binding elements necessary for efficient protease/substrate interactions. In the case of the *B. anthracis* lethal factor protease used in microsphere-based protease studies optimized cleavage sites have been developed that cut efficiently enough for use in microsphere assays (Saunders, et al., 2006; Cummings, et al., 2002; Turk, et al., 2004). In the case of *C. botulinum* light chain protease assays, we have used full length protease substrates, which provide optimal cleavage when compared to cleavage sites alone or substrates with distal binding elements deleted (Saunders, et al., 2010). Nonetheless, for novel target substrates, it should not be assumed that short amino acid sequences that mimic natural cleavage sites of proteases alone will be sufficient for cleavage of substrates *in vitro*. Substrate optimization methods may be needed to determine optimal peptide sequences for use in *in vitro* assays (Diamond, 2007). Use of full length protease substrates is likely to provide better results and has also been shown to give faster proteolytic rates than cleavage sites alone in these assays.

Lack of reproducible protease assays in high-throughput screening—

Adaptation of microsphere-based protease assays to high throughput screening platforms

will require optimization. Typical variables in these assays leading to a lack of protease cleavage and acceptable Z' factors (above 0.5) include protease concentration or incubation times. Proteases in solution will lose their activity over time. If the protease under investigation loses activity quickly, a small change of fluorescence will be seen regardless of incubation time. This can be tested by evaluating microsphere-based protease assays in non high-throughput assays measuring cleavage over time either from the surface of microspheres as described in Basic Protocol, or by fluorimetric measurements such as FRET assays. If the protease activity is lost quickly, increasing the amount of protease in the sample wells will permit shorter incubation times. When a protease is stable over extended periods of time (2+ hours), increased incubation times can be used to augment cleavage from the surface of microspheres.

Anticipated results

Once optimal microsphere and protease concentrations are established, it is expected that a loss of fluorescence will yield a pseudo-first order exponential decay. Fluorescence will likely not reach background (auto-fluorescence of the microspheres) as some of the substrate may not be accessible to the protease or because of non-specific binding of substrate to the microsphere, where cleavage events will not lead to loss of fluorescence. Fluorescence can be normalized by dividing all mean or median fluorescence time point measurements by the mean or median fluorescence measurement prior to protease addition. This is particularly useful when analyzing multiple substrates or proteases at once using multiplex microsphere sets to give relative rates of cleavage. This will give a range of normalized fluorescence from 0 to 1 and approximate percentage of starting substrate present on the microsphere at certain time points, this can be determined by multiplying the normalized fluorescence value by 100. Subtracting the percentage of substrate present from 100 will give a rough estimate of percentage of substrate cleaved.

Using no protease and no compound control samples, test compounds or known inhibitors can be shown to inhibit proteases of interest. Percent inhibition of a tested concentration of an inhibitor can be calculated using the percentage inhibition formula in step 14 of Support Protocol 3. Positive controls will be no protease and/or a well with a high concentration of a known inhibitor. Negative controls will be no test compound with protease. The same concentration of protease will need to be used in the negative control and all inhibitor containing samples in order to determine percentage inhibition using this formula.

It should be noted that traditional steady state kinetics models to calculate K_m and V_{max} from these microsphere based assays cannot be done due to the low concentration of substrate used in these assays, which results in $[S]$ being $\ll [E]$ and makes typical steady state assumptions invalid (Copeland, 2000). $[S]$ can be determined simply by using FITC and GFP standard microspheres to generate a standard curve to estimate the number of fluorescent molecules on the surface of the microsphere, multiplying by the estimated number of microspheres, and dividing by the volume (typically resulting $[S]$ in the picomolar range).

Time considerations

Microsphere-based protease assays usually include a one hour binding step, followed by 10 to 15 minutes for wash steps, as well as the assay time. Protease assays should be performed continuously until the entire sample is aspirated into the flow cytometer. Samples of 500 μ l can typically be acquired for 30 minutes depending on the flow cytometer and flow rate. For measurements at defined time points, for example every 15 minutes or every hour, the assay can be performed until the protease loses activity or the maximum amount of cleavage from the microsphere occurs. For HTS, one hour binding times are followed by compound assay plate setup (this can be quick using robotics, very slow by hand), optimized incubation times, readout and data analysis. Times (2–12 hours) will also depend on the number of microplates sampled.

Acknowledgments

This work was supported by the National Flow Cytometry Resource NIH grant RR001315, University of New Mexico Center for Molecular Discovery MH077425 and MH084690, and Joint Sciences Technologies Laboratories grant (JSTL) 26Q4.

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Figure 13.12.1.

A. Protease substrates are designed to have purification and attachment chemistry (*in vivo* biotinylation tag) at one end, and a fluorescent protein (GFP) at the other end with a protease cleavage site or full length protein substrate sub-cloned in-frame between them. **B.** Purified biotinylated protease substrates are bound to streptavidin coated microspheres and the protease of interest is added. For simplicity, only one protein is shown bound to a microsphere (not to scale), whereas in the experimental conditions each microsphere will have between 100,000 and 1 million fluorescent substrate molecules bound as determined by GFP and FITC standard microsphere sets. Cleavage of the protease substrate occurs and is measured as a loss of fluorescence from the surface of the microspheres via flow cytometry.

Figure 13.12.2.

Biotinylated GFP protease substrate titration onto streptavidin coated microspheres (Spherotech Inc.). **A** ppGFP, a protease substrate for the protease factor Xa, bound in increasing amounts to streptavidin coated microspheres blocked with biotin and unblocked streptavidin coated microspheres. Specific binding (green) is determined by subtracting the mean green fluorescence of the blocked samples (red) from the unblocked samples (black). This protein preparation had very low non-specific binding. **B** Protein titration for biotinylated SNAP-25 GFP, a substrate for the *Botulinum* neurotoxin type A light chain protease, onto streptavidin coated microspheres. This protein has more non-specific binding than the ppGFP protein in A (above) but has acceptable levels of specific binding. Optimal

substrate binding conditions for these proteins in protease assays would be ~ 100 nM for ppGFP (A) and 300 nM for SNAP-25 (B)

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Figure 13.12.3.

Sample gating of microspheres for analysis of fluorescence and protease cleavage over time.

A. Bivariate plot of forward and size scatter with single microsphere population gated. **B.** Histogram of green fluorescence (FL1, 530/15 nm) of size-gated microsphere population from A. **C.** Histogram plot of four multiplex microspheres fluorescent in the FL2 channel (585/21/ nm). Gates are drawn on this histogram plot for each microsphere type. The small secondary peak populations for each microsphere are common in multiplex microsphere sets and can be gated out in the FL2 histogram. **D.** Fluorescence vs. time for one size, and gated microsphere as gated in A and C. No protease was added in this sample. All data analysis for these sample gates was done by exporting files from an Accuri C6 flow cytometer as FCS files and plotted graphically in FlowJo flow cytometry data analysis software.

Figure 13.12.4.

Normalized fluorescence of cleavage of biotinylated SNAP-25 GFP from the surface of streptavidin coated microspheres by the *Botulinum* Neurotoxin type A Light Chain protease (BoNT/A LC). Each trace is from a separate 500 μ l sample of microspheres in protease buffer with the indicated amount of BoNT/A LC added after 60 seconds of acquisition of microspheres on an Accuri C6 flow cytometer. Data were analyzed on HyperView IDL software written by Dr. Bruce Edwards calculating the median FL1 fluorescence of microspheres every 0.3 seconds. Data were normalized by averaging the first 60 second median FL1 values and dividing that average and subsequent median FL1 values for that sample by that value.