



## **UbiQ protocol P006 \_ Fluorescence-based proteasome activity profiling**

description: profiling of proteasomal activity in cell lysates, intact cells, and murine and human patient-derived material, with high sensitivity using SDS-PAGE, proteasome activity-based probe UbiQ-018 (<https://ubiqbio.com/product/ubiq-018/>)

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# Chapter 13

## Fluorescence-Based Proteasome Activity Profiling

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### Abstract

With the proteasome emerging as a therapeutic target for cancer treatment, accurate tools for monitoring proteasome (inhibitor) activity are in demand. In this chapter, we describe the synthesis and use of a fluorescent proteasome activity probe that allows for accurate profiling of proteasomal activity in cell lysates, intact cells, and murine and human patient-derived material, with high sensitivity using SDS-PAGE. The probe allows for direct scanning of the gel for fluorescent emission of the distinct proteasomal subunits and circumvents the use of Western blot analysis. Due to its suitable biochemical and biophysical properties, the fluorescent probe can also be used for confocal laser scanning microscopy and flow cytometry-based experiments.

**Key words:** Proteasome, Inhibition, Activity profiling, Fluorescent probe, Proteasome activity assay

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### 1. Introduction

The use of proteasome inhibitors in the clinic for cancer treatment (1) has validated the proteasome as therapeutic target. The proteasome is responsible for the degradation of misfolded and redundant proteins and of key regulatory proteins, involved in many cellular processes such as proliferation and survival (2, 3). Inhibition of the proteasome causes disruption of many of these processes, eventually leading to cell death (1, 4). The proteasome inhibitor bortezomib (1, 5) (Fig. 1) is currently used for the treatment of multiple myeloma (6) and mantle cell lymphoma (7). A number of second-generation proteasome inhibitors, which differ in their mode of inhibition and subunit specificity, are currently in clinical trials. Eukaryotic 26S proteasomes consist of a 20S core and one or two 19S regulatory caps. The 19S regulatory caps are involved in

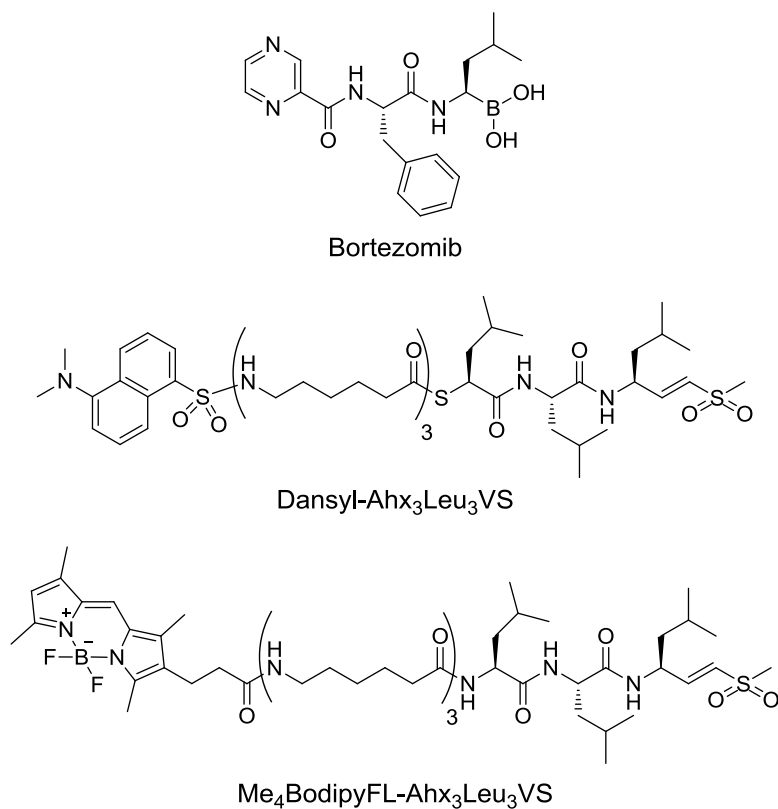


Fig. 1. Structures of bortezomib, dansyl-Ahx<sub>3</sub>Leu<sub>3</sub>VS, and Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS.

the recognition and unfolding of polyubiquitinated substrates, while the catalytic activity takes place in the 20S core (3). The 20S proteasome is a barrel-shaped protein complex composed of four stacked rings that consist of seven subunits each. The two outer rings are made up of  $\alpha$ -subunits and interact with the 19S regulatory caps. The two inner rings consist of  $\beta$ -subunits, from which three constitutive subunits, termed  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5, are responsible for proteolysis. The  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 subunits each provide a distinct protease activity, the caspase-like (cleavage after acidic residues), tryptic-like (cleavage after basic residues), and chymotryptic-like (cleavage after hydrophobic residues) activity, respectively. Upon interferon- $\gamma$  stimulation, three additional immunoproteasome subunits ( $\beta$ 1i,  $\beta$ 2i, and  $\beta$ 5i) are expressed, which replace the constitutive  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 subunits to form the immunoproteasome. Immunoproteasomes are thought to have altered catalytic activity favoring production of antigenic peptides and are mainly expressed in lymphoid tissues, e.g., spleen, thymus, and lymph nodes (8). Different cells can express different ratios of constitutive and immunoproteasome subunits (9, 10). Variations in proteasomal composition affect substrate specificity and sensitivity to proteasome

inhibition. To predict the sensitivity of patients to proteasome inhibitors, accurate tools are required that can correlate proteasome composition and the extent of proteasome inhibition to treatment response. Reagents that can be used to profile proteasome activity are valuable research tools and hold promise as diagnostic reagents.

Techniques that are commonly used to monitor proteasome activity include the application of fluorogenic substrates (11, 12), small molecule-based activity assays (13–18), and models based on recombinant reporter proteins (19–21). Traditionally, fluorogenic substrates are used to measure the activity of different proteasome active sites, but most fluorogenic substrates cannot be used in cells, and prior cell lysis is required before activity measurements can be performed. Reporter proteins can be used in living cells, but their use is limited to genetically altered cells or organisms. In addition, their activity readout depends on the balance between synthesis and degradation of fusion proteins, which involves many cellular factors other than the proteasome, including the rate of fusion-protein synthesis.

The first small molecule-based activity assay for profiling of the specificity of proteasome inhibitors in living cells was reported in 2005 (13). This dansylated vinylsulfone based probe (Fig. 1) contains a proteasome-targeting motif,  $\alpha,\beta$ -unsaturated vinyl sulfone (VS) group that covalently reacts through a Michael addition with the  $\gamma$ -hydroxyl of the N-terminal threonine residue of all catalytic  $\beta$ -subunits of the proteasome (22), resulting in the formation of a  $\beta$ -sulfonyl ether linkage. Antibodies against the dansyl moiety were used for the detection of labeled active subunits by Western blot analysis (13). Subsequent replacement of the dansyl group by high quantum yield fluorophores allowed for direct scanning of the SDS-PAGE gel for fluorescence emission of fluorescently labeled subunits (14, 18). Due to their favorable biochemical and biophysical properties, Bodipy-based proteasome activity probes can be used to monitor proteasome activity in cell extracts, living cells, and murine tissues using a range of techniques including SDS-PAGE (14), confocal laser scanning microscopy (14, 18), and flow cytometry (14). The advantage of flow cytometry-based assays is that they can be used to measure both proteasome activation and proteasome inhibition in large numbers of samples. Information on the activity of distinct subunits on the other hand can only be obtained in SDS-PAGE-based profiling experiments.

A typical SDS-PAGE-based profiling experiment is shown in Fig. 2. Lysates of MelJuso (human melanoma; Fig. 2a) and THP-1 (Human monocytic leukemia, Fig. 2b) cells were incubated with the proteasome inhibitors MG132 (25  $\mu$ M), bortezomib (0.1  $\mu$ M and 1  $\mu$ M), epoxomicin (1  $\mu$ M), or a DMSO control. Subsequently, samples were incubated with fluorescent proteasome activity probe to label proteasome subunits, proteins were separated by

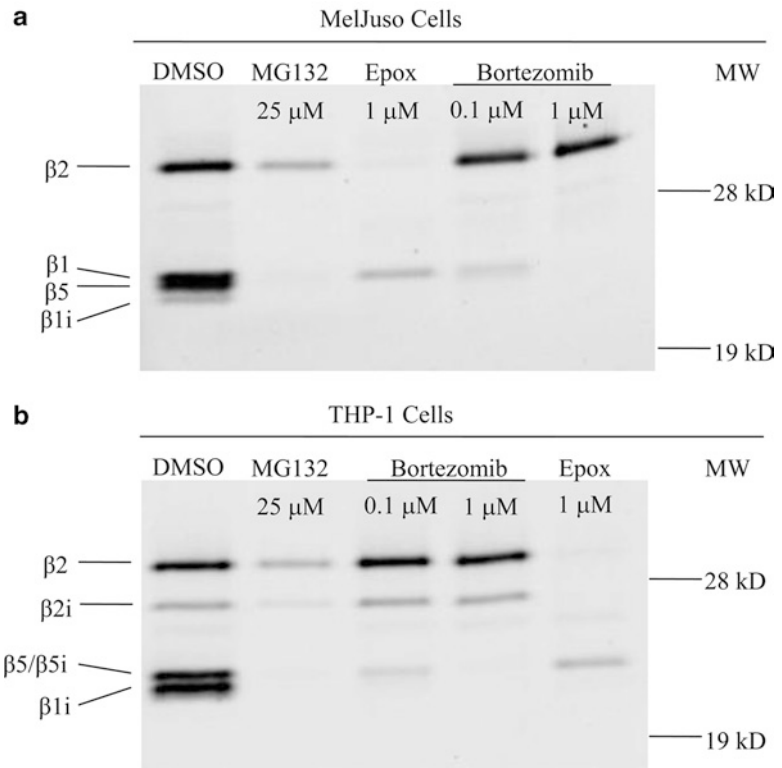


Fig. 2. Gel images showing the active proteasome subunit labeling in MelJuso (**a**) and THP-1 (**b**) cell lysates that were preincubated with the indicated concentrations of MG132, epoxomicin or bortezomib, followed by incubation with 1 μM Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS.

SDS-PAGE and the resulting gel was scanned for fluorescence emission. Upon incubation of proteasome with probe, all active proteasome subunits become fluorescently labeled. Prior inhibition of a subunit with a proteasome inhibitor prevents probe binding, resulting in the disappearance of a fluorescent band on the gel. Therefore, the measured fluorescence intensity directly correlates to the activity of the labeled β-subunit. In the DMSO controls, all active subunits are labeled, and the composition of proteasome in these cells was visualized (Fig. 2). Incubation with different proteasome inhibitors resulted in the disappearance of particular bands on the gels, indicative of the subunit specificity of these inhibitors.

Figure 3 shows the results of a typical fluorescence assisted cell sorting (FACS) assay, in which the fluorescence intensity is plotted versus the cell count. MelJuso cells were incubated with 1 μM MG132, followed by incubation with probe (Fig. 3, blue curve). As controls, cells were incubated with probe only (Fig. 3, red curve) or not incubated (Fig. 3, black curve). Addition of probe results in a shift of the cell population toward higher fluorescence intensity. Upon preincubation with MG132, the peak shifts back to lower fluorescence intensity, indicating inhibition. As some

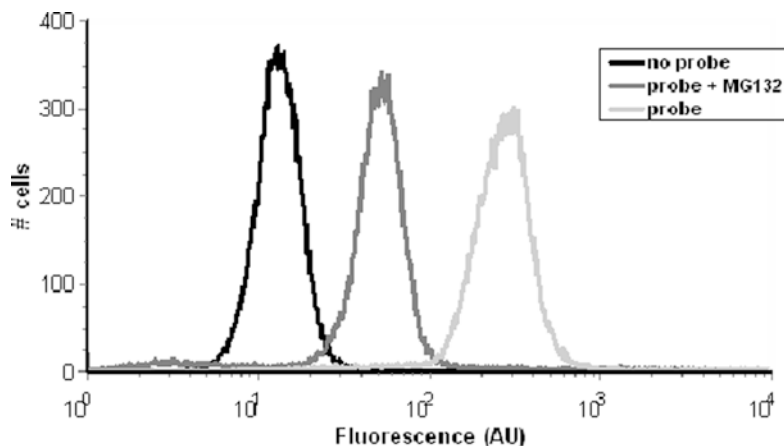


Fig. 3. Flow cytometry histograms showing the fluorescence intensity in MeJuso cells that were nonincubated (*black*), incubated with 200 nM Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS (*red*), or preincubated with 1 μM MG132, followed by incubation of 200 nM Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS (*blue*).

probe always aspecifically sticks to cell membranes, the signal intensity at 1 μM MG132 is taken as 100% inhibition, whereas the signal intensity in probe only treated cells is taken as 0% inhibition.

In this chapter, we describe the synthesis of the Bodipy-based fluorophore Me<sub>4</sub>BodipyFL-*N*-hydroxy-succinimidyl (NHS) ester in six steps. The Bodipy fluorophore was chosen for its ease of synthesis from commercially available reagents on a large scale and for its fluorescein-like spectral properties (excitation maximum of 515 nm and emission maxima of 519 nm), which make it widely applicable to common fluorescence-based methodologies. In addition, we describe the synthesis of the proteasome-targeting moiety Ahx<sub>3</sub>Leu<sub>3</sub>VS and subsequent coupling with Me<sub>4</sub>BodipyFL-NHS ester yielding the fluorescent probe Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS (Fig. 1). Finally, optimized procedures to profile the effects of proteasome inhibitors in cell lysates, living cells, and murine tissues using this fluorescent proteasome activity probe in both SDS-PAGE and FACS-based assays are described.

## 2. Materials

### 2.1. Synthesis of the Fluorescent Probe Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS

#### 2.1.1. Synthesis of Me<sub>4</sub>BodipyFL-NHS Ester

1. *Tert*-butyl acetoacetate, acetic acid, sodium nitrite.
2. Methyl 4-acetyl-5-oxohexanoate, zinc dust, sodium acetate, ethyl acetate (EtOAc), magnesium sulfate (MgSO<sub>4</sub>).
3. Trifluoroacetic acid (TFA), triethylorthoformate, toluene, EtOAc.
4. Ethanol (absolute), 2,4-dimethylpyrrole, HCl in dioxane (4 N), diethyl ether.

5. 1,2-Dichlorobenzene, triethylamine, boron trifluoride etherate, hexanes, EtOAc, toluene.
6. 1 M Lithium hydroxide solution, EtOAc, 0.1 M HCl, MgSO<sub>4</sub>, acetic acid.
7. Dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), *N*-hydroxysuccinimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), 4-dimethylaminopyridine (DMAP), hexanes, EtOAc.

### 2.1.2. Synthesis of Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS

1. Fmoc-L-leucine-PEG-polystyrene resin (Applied Biosystems), Fmoc-leucine-OH (Fmoc-Leu-OH, Novabiochem), Fmoc-aminohexanoic acid (Fmoc-Ahx-OH, Novabiochem), piperidine, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop, coupling reagent), *N,N*-diisopropylethylamine (DIPEA), *N*-methyl-2-pyrrolidone (NMP).
2. Di-*tert*-butyl-dicarbonate, DIPEA, dimethylformamide (DMF), CH<sub>2</sub>Cl<sub>2</sub>, methanol (MeOH), acetic acid.
3. (*S*;*E*)-5-methyl-1-(methylsulfonyl)hex-1-en-3-amine (leucinyln vinyl sulfone, LeuVS); EDCI; DIPEA; DMF; CH<sub>2</sub>Cl<sub>2</sub>; MeOH.
4. TFA; toluene; diethyl ether.
5. Me<sub>4</sub>-BodipyFL-NHS ester (7); DIPEA; DMF.

### 2.1.3. Analytical Procedures

1. Acros silica gel (0.030–0.075 mm) for flash chromatography.
2. Silica-coated plastic sheets (Merck silica gel F<sub>254</sub>) for thin-layer chromatography (TLC).
3. LC/MS analysis: Waters LCT mass spectrometer in line with a Waters 2795 HPLC system and a Waters 2996 photodiode array detector. Reversed-phase runs were performed on a 3 μm Atlantis T3, C18 RP, 2.1 × 100 mm column (Waters) using gradient elution with H<sub>2</sub>O/0.1% formic acid as solvent A and acetonitrile/0.1% formic acid as solvent B at a flow rate of 0.4 mL/min.
4. Preparative HPLC: Waters 1525 EF HPLC system in line with a Waters 2487 dual λ absorbance detector using gradient elution with H<sub>2</sub>O/0.05% TFA as solvent A and acetonitrile/0.05% TFA as solvent B. Preparative runs were performed on a 10 μm 19 × 250 mm Atlantis dC18 column (Waters) at a flow rate of 18 mL/min.
5. Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance 300 (<sup>1</sup>H: 300 MHz; <sup>13</sup>C: 75 MHz) spectrometer.
6. Fluorescence spectra were measured on a customized fluorimeter set-up using a mercury vapor lamp at 72 W, appropriate gratings, and a photomultiplier at 1,000 V (Photon Technology International). Excitation spectra were recorded at 100 nM in water at 550 nm emission, while emission spectra were recorded in water at 480 nm excitation.

## **2.2. Profiling of Proteasome Activity Using SDS-PAGE-Based Assays**

### *2.2.1. In Vitro Profiling of Proteasome Activity in Cell Lysates*

1. Cell line of choice cultured in appropriate medium, e.g., DMEM (Dulbecco's modified Eagle's medium) for adherent cell lines, and RPMI 1640 (Roswell Park Memorial Institute) medium for suspension cell lines supplied with fetal calf serum (FCS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin).
2. Phosphate-buffered saline (PBS), Trypsin solution (0.25%, Invitrogen) for adherent cells.
3. HR lysis buffer (see Note 1): 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 250 mM sucrose, 1 mM DTT (added fresh from a 1 M stock solution before use), 2 mM ATP (added fresh from a 0.5 M stock solution before use). Prepare HR buffer lacking DTT and ATP, filter over a 0.22 µm filter (e.g., MILLEX®GS, Millipore), and store at 4°C. Supplement the amount of HR buffer needed for a single experiment (typically 1 mL) with ATP and DTT before use (see Note 2).
4. Bradford reagent (Biorad).
5. Dimethylsulfoxide (DMSO) (optional).
6. 50× Stock solutions of proteasome inhibitors in DMSO. Store at -20°C.
7. 50 µM Stock solution of Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS in DMSO. Store at -20°C.

### *2.2.2. Labeling of Active Proteasome Subunits in Living Cells*

1. Cell line of choice cultured in appropriate medium (see Subheading 2.2.1).
2. 500× Stock solutions of proteasome inhibitors in DMSO. Store at -20°C.
3. 50 µM Stock solution of Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS in DMSO. Store at -20°C.
4. 5 mM Stock solution of MG132 (Sigma) in DMSO. Store at -20°C.
5. PBS, Trypsin solution (0.25%, Invitrogen) for adherent cells.
6. NP40 lysis buffer (see Note 1): 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40. Prepare NP40 lysis buffer, filter over a 0.22 µm filter (e.g., MILLEX®GS 0.22 µm Filter unit, Millipore), and store at 4°C.
7. Bradford reagent (Biorad).

### *2.2.3. Ex Vivo Profiling of Proteasome Subunit Activity in Murine Tissues*

1. Freshly dissected mouse tissue of choice.
2. HR lysis buffer (see item 3 in Subheading 2.2.1).
3. Glass beads (≤106 µm, acid washed, Sigma).
4. Bradford reagent (Biorad).
5. 50 µM Stock solution of Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS in DMSO. Store at -20°C.



#### 2.2.4. Gel Electrophoresis and In-Gel Fluorescence Readout

1. Precast gel system (NuPAGE, Invitrogen).
2. NuPAGE® Novex 12% Bis-Tris Gel, 1.0 mm (Invitrogen), NuPAGE® MOPSSDS Running buffer (Invitrogen), NuPAGE® Antioxidant (Invitrogen), SeeBlue® Plus2 Pre-Stained Standard (Invitrogen).
3. 3× Reducing sample buffer (see Note 1). For 1.2 mL: 900 μL 4× NuPAGE® LDS Sample buffer (Invitrogen), 90 μL β-mercaptoethanol (Sigma), 210 μL water. Store at room temperature.
4. ProXPRESS 2D Proteomic imaging system (Perkin Elmer) for the determination of In-gel fluorescence intensity. Fluorescence intensities are quantified using the analysis software TotalLab.

#### 2.3. Flow Cytometry

1. MelJuso cells (human melanoma) cultured in DMEM supplied with FCS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin).
2. 96-Well flat bottom tissue culture plates (BD Falcon).
3. DMSO, PBS, Trypsin solution (0.25%; Invitrogen).
4. 2.5 mM Stock solutions of the desired compounds in DMSO. Store at -20°C.
5. 0.4 mM Stock solution of MG132 (Sigma) in DMSO. Store at -20°C.
6. 50 μM Stock solution of Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS in DMSO. Store at -20°C.
7. FACS buffer: PBS containing 2% FCS. Store at 4°C.
8. Fixation buffer: PBS containing 2% formaldehyde. Store at room temperature.
9. FACSCalibur (BD Biosciences, 488 nm laser).

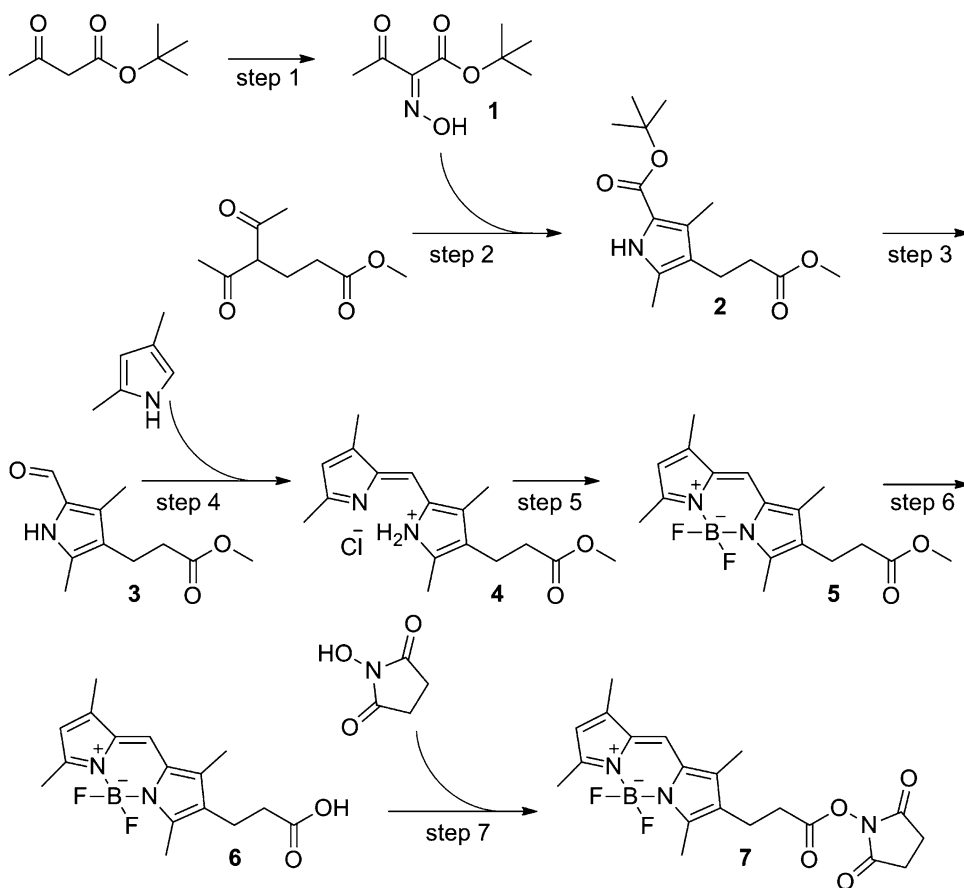
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### 3. Methods

#### 3.1. Synthesis of the Fluorescent Proteasome Probe Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS

##### 3.1.1. Synthesis of Me<sub>4</sub>-BodipyFL-NHS Ester (Scheme 1)

1. To a mixture of *tert*-butyl acetoacetate (2.80 g, 17.4 mmol) in acetic acid (4 mL), stirred on an ice bath, add dropwise a solution of sodium nitrite (1.40 g, 20.3 mmol) in deionized water over a 5 min period. Stir for an additional 16 h at 4°C (e.g., in a cold room) (23).
2. Slowly, add the resulting solution containing oxime 1 in five portions to a solution of methyl 4-acetyl-5-oxohexanoate (5 mL, 28.6 mmol) in acetic acid (15 mL) kept at 65°C. Simultaneously, add a mixture of zinc dust (3 g, 45.9 mmol)



Scheme 1. Synthesis of Me<sub>4</sub>BodipyFL-NHS ester 7. Step numbers correspond to step numbers outlined in Subheading 3.1.1

and sodium acetate (3 g, 36.6 mmol) in five portions. Stir the mixture for 2 more hours at 65°C, then pour it into ice water (250 mL) and leave slowly stirring for 16 h, allowing the mixture to warm up to room temperature. Filter off the precipitate, including product and zinc residues. Take up the residue in EtOAc (100 mL) and dry the organic layer with MgSO<sub>4</sub>. Filter off the MgSO<sub>4</sub> and concentrate the filtrate in vacuo to dryness to yield tetrasubstituted pyrrole 2 as a light brown solid (2.87 g, 10.2 mmol, 59% yield). The crude product can be used without further purification in the next step.

- Dissolve pyrrole 2 (1 g, 3.55 mmol) in TFA (20 mL) and stir for 20 min on ice. Slowly, add triethylorthoformate (2 mL, 12 mmol) and stir the solution gently at 0°C for 15 min. Add water (2 mL) and stir for 10 min at 0°C. Coevaporate the solution with toluene (60 mL) in vacuo to dryness at room temperature.

Purify the crude product by flash column chromatography (24) using EtOAc as the eluent to yield aldehyde **3** as a brown solid (469 mg, 2.25 mmol, 63% yield).

- Dissolve aldehyde **3** (469 mg, 2.25 mmol) in absolute ethanol (8 mL) and cool to 0°C. Add 2,4-dimethylpyrrole (256 mg, 2.69 mmol) under an argon atmosphere and add 4 mL of cold 4 N HCl in dioxane. Stir for 15 min at 0°C and then transfer the solution to a 50-mL Falcon tube and centrifuge at 1,500 × *g* for 10 min at 4°C. Discard the supernatant, resuspend the resulting orange solid in cold diethylether (40 mL), and centrifuge again. Decant the supernatant and dry the residue under a stream of nitrogen to yield crude dipyrrole **4** as an orange solid, which is used in the next step without further purification.
- Suspend dipyrrole **4** in 1,2-dichlorobenzene (20 mL) under an argon atmosphere and add triethylamine (1 mL, 7.16 mmol) and boron trifluoride diethyl etherate (1 mL, 8.12 mmol) in concert. Stir the resulting metallic solution for 30 min at 100°C and then allow the mixture to cool to room temperature. Dilute the solution with dry hexanes (25 mL) and apply directly onto a silica gel column for purification by flash column chromatography using 25% EtOAc in toluene as the eluent to afford Me<sub>4</sub>BodipyFL-methyl ester **5** as an orange solid (481 mg, 1.44 mmol, 64% yield starting from aldehyde **3**).
- Dissolve Me<sub>4</sub>BodipyFL-methyl ester **5** (400 mg, 1.24 mmol) in ethanol (6 mL) and add a solution of 1 M lithium hydroxide in water (1.3 mL). Stir the solution until monitoring by TLC (eluent: EtOAc containing 0.1% acetic acid) reveals complete conversion. Add EtOAc (40 mL) and 0.1 M HCl (30 mL) and separate the two layers. Dry the organic layer with MgSO<sub>4</sub> and concentrate in vacuo to dryness to obtain an orange solid.
- Purify by flash column chromatography using EtOAc containing 0.1% acetic acid as the eluent to yield Me<sub>4</sub>BodipyFL-OH **6** as an orange solid (306 mg, 0.95 mmol, 77% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.09 (s, 1H), 6.10 (s, 1H), 2.66 (dd, *J* = 8.2 Hz, *J* = 7.1 Hz, 2H), 2.59 (s, 6H), 2.60–2.53 (m, 2H), 2.31 (s, 3H), 2.27 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 178.2 (CO), 156.5 (C<sub>q</sub>), 155.3 (C<sub>q</sub>), 141.0 (C<sub>q</sub>), 138.1 (C<sub>q</sub>), 133.3 (C<sub>q</sub>), 132.6 (C<sub>q</sub>), 127.8 (C<sub>q</sub>), 119.7 (CH), 118.9 (CH), 33.9 (CH<sub>2</sub>), 19.2 (CH<sub>2</sub>), 14.6 (CH<sub>3</sub>), 12.7 (CH<sub>3</sub>), 11.2 (CH<sub>3</sub>), 9.6 (CH<sub>3</sub>). Fluorescence data: λ<sub>ex</sub> = 515 nm, λ<sub>em</sub> = 519 nm, see Fig. 4.
- Dissolve Me<sub>4</sub>BodipyFL-OH **6** (306 mg, 0.95 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (60 mL) and add *N*-hydroxy succinimide (120 mg, 1.05 mmol), coupling reagent EDCI (201 mg, 1.05 mmol), and coupling catalyst DMAP (11 mg, 0.1 mmol). Stir the mixture for 16 h at room temperature and add 0.05 M HCl (40 mL) and separate the two layers. Dry the organic layer with MgSO<sub>4</sub> and concentrate in vacuo to dryness to obtain an orange solid.

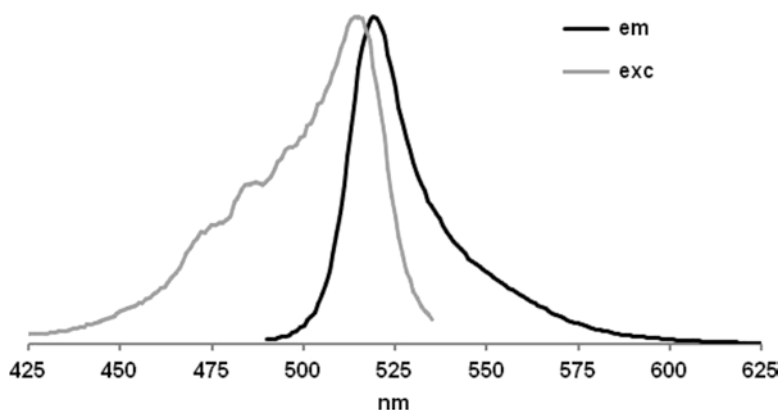
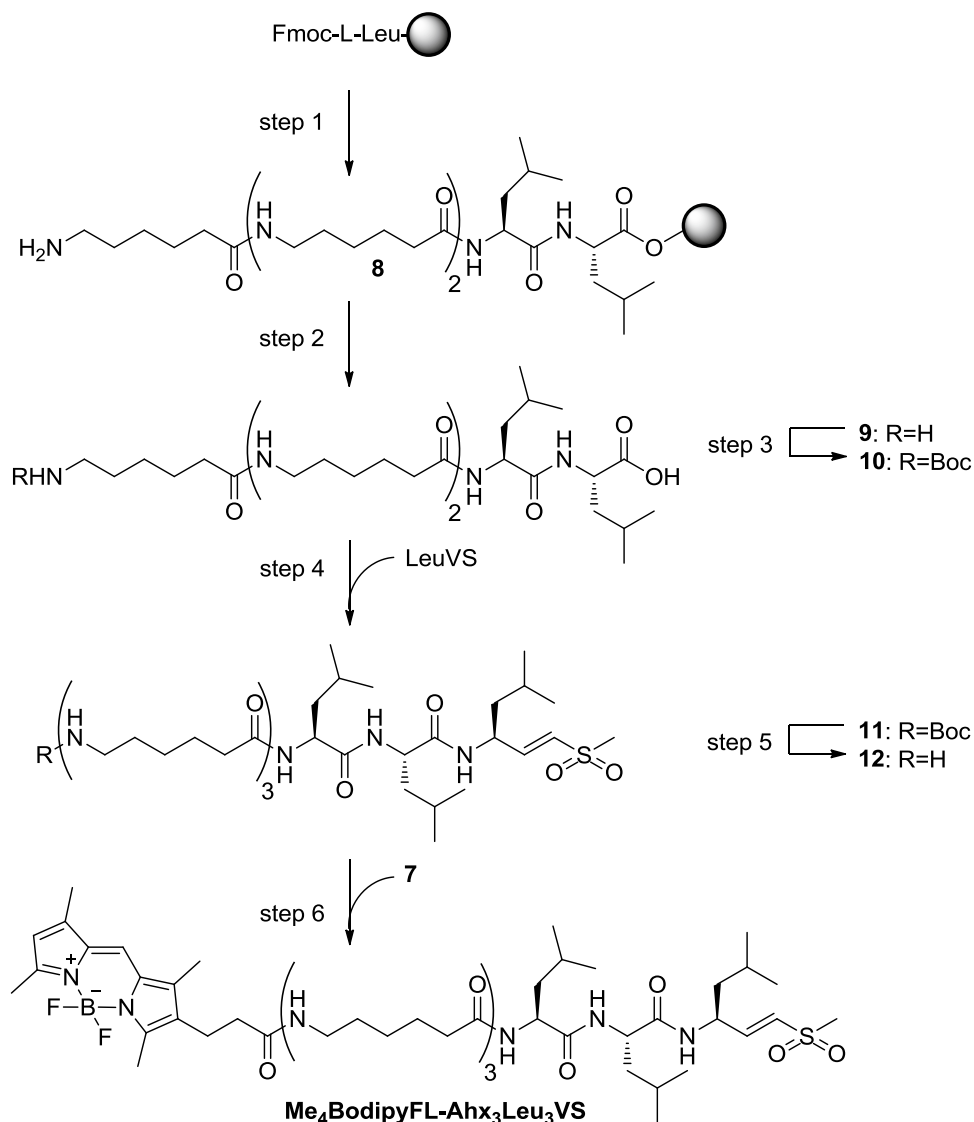


Fig. 4. Fluorescence spectrum of  $\text{Me}_4\text{BodipyFL-OH}$  measured in water, displaying  $\lambda_{\text{exc}} = 515$  nm and  $\lambda_{\text{em}} = 519$  nm. Derivatives of this dye display similar fluorescence spectra.

- Purify by flash column chromatography using EtOAc:hexanes (2:3 v/v) as the eluent to obtain  $\text{Me}_4\text{BodipyFL-NHS}$  ester **7** as a dark orange solid (242 mg, 0.85 mmol) in 61% yield.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.02 (s, 1H), 6.02 (s, 1H), 2.85–2.78 (m, 6H), 2.75–2.70 (m, 2H), 2.50 (s, 6H), 2.22 (s, 3H), 2.19 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  169.0 (2 $\times$  CO), 167.7 (CO), 156.9 ( $\text{C}_q$ ), 154.8 ( $\text{C}_q$ ), 141.3 ( $\text{C}_q$ ), 138.0 ( $\text{C}_q$ ), 133.4 ( $\text{C}_q$ ), 132.5 ( $\text{C}_q$ ), 126.7 ( $\text{C}_q$ ), 119.9 (CH), 119.0 (CH), 31.1 ( $\text{CH}_2$ ), 25.6 (2 $\times$   $\text{CH}_2$ ), 19.2 ( $\text{CH}_2$ ), 14.6 ( $\text{CH}_3$ ), 12.6 ( $\text{CH}_3$ ), 11.2 ( $\text{CH}_3$ ), 9.6 ( $\text{CH}_3$ ). Fluorescence data:  $\lambda_{\text{ex}} = 513$  nm,  $\lambda_{\text{em}} = 517$  nm.

### 3.1.2. Synthesis of $\text{Me}_4\text{BodipyFL-Ahx}_3\text{Leu}_3\text{VS}$

- The synthesis of  $\text{Me}_4\text{BodipyFL-Ahx}_3\text{Leu}_3\text{VS}$  is depicted in Scheme 2 (see Note 3). Fmoc-L-Leu-PEG-polystyrene Wang resin (1 g, 0.17 mmol equivalents) (see Note 4) is subjected to four coupling cycles, in which deprotection of the Fmoc group with piperidine/NMP (1:4 v/v; 10 mL/g of dry resin) is followed by a coupling cycle with three equivalents of Fmoc-protected amino acid, three equivalents of DIPEA, and three equivalents of PyBop coupling reagent in NMP (10 mL/g of dry resin). After each step, the resin is thoroughly washed with NMP (5  $\times$  10 mL). Coupling steps are performed with Fmoc-Leu-OH (1 $\times$ ) and Fmoc-Ahx-OH (3 $\times$ ), sequentially (see Note 5) (**14**).
- After the final coupling step, remove the Fmoc group with piperidine/NMP (1:4 v/v), to afford solid supported **8**, and subsequently cleave the peptide from the resin by treating the resin with 100% TFA (10 mL) for 30 min. Coevaporate the cleavage mixture with toluene (40 mL) to obtain  $\text{H}_2\text{N-Ahx}_3\text{Leu}_2\text{-OH}$  **9** (100 mg, 0.17 mmol) in >90% purity as judged by LC/MS analysis. The product is used in the next step without further purification.



Scheme 2. Synthesis of Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS. Step numbers correspond to step numbers outlined in Subheading 3.1.2.

3. Dissolve H<sub>2</sub>N-Ahx<sub>3</sub>Leu<sub>2</sub>-OH **9** (100 mg, 0.17 mmol) in DMF (2 mL) and add DIPEA (148 μL, 0.85 mmol) and di-*tert*-butyl-dicarbonate (44 mg, 0.20 mmol). Stir the resulting suspension for 16 h at room temperature and then concentrate in vacuo to dryness to yield an off-white solid. Purify by flash column chromatography using a gradient of 10–20% MeOH in CH<sub>2</sub>Cl<sub>2</sub> containing 0.1% acetic acid as the eluent to obtain Boc-Ahx<sub>3</sub>Leu<sub>2</sub>OH **10** as a white solid (116 mg, 0.17 mmol) in >98% yield.
4. L-Leucinyl vinyl sulfone (LeuVS) was prepared as reported ([22](#), [25–27](#)). Dissolve LeuVS (50 mg, 0.26 mmol) in DMF

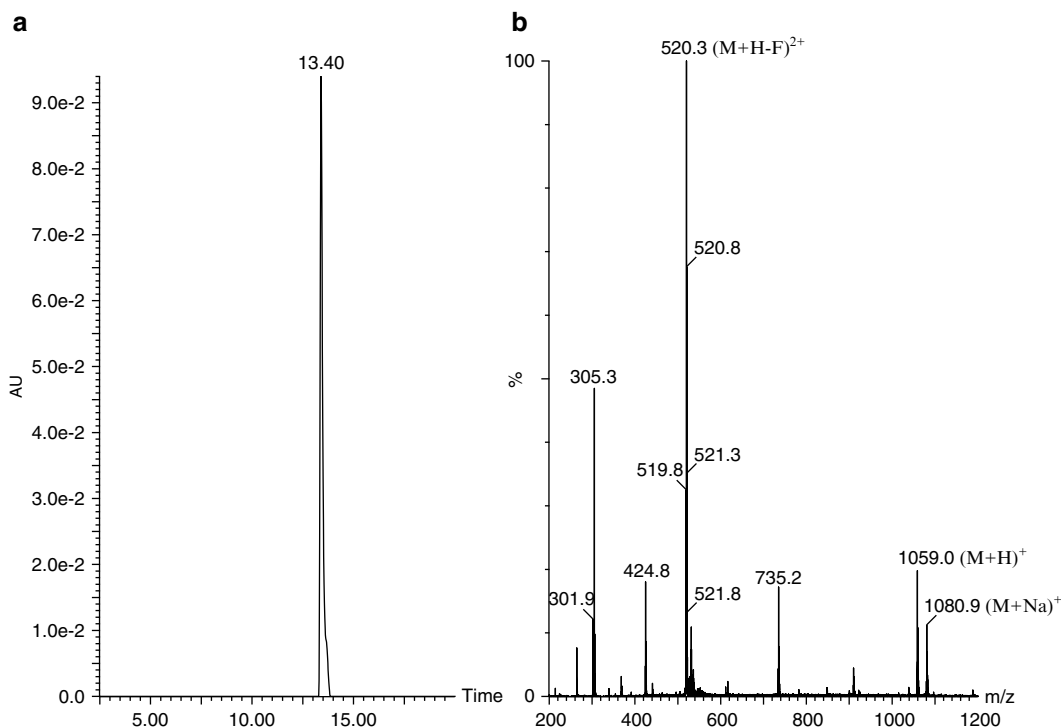


Fig. 5. Reversed-phase chromatogram (a) and MS spectrum (b) of Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS. MS (ESI): 1,058.66 (M+H)<sup>+</sup>; 520.27 (M+H-F)<sup>2+</sup>; 1,080.91 (M+Na)<sup>+</sup>.

and add Boc-Ahx<sub>3</sub>Leu<sub>2</sub>OH **10** (116 mg, 0.17 mmol), DIPEA (89 μL, 0.51 mmol), and coupling reagent EDCI (50 mg, 0.26 mmol). Stir the solution for 3 h at room temperature and then concentrate in vacuo to dryness to yield an off-white solid. Purify by flash column chromatography using 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> as the eluent to obtain Boc-Ahx<sub>3</sub>Leu<sub>3</sub>VS **11** as a white solid (103 mg, 0.12 mmol) in 71% yield.

5. In a 50-mL Falcon tube, dissolve Boc-Ahx<sub>3</sub>Leu<sub>3</sub>VS **11** (12 mg, 14 μmol) in TFA (1 mL) and leave at room temperature for 30 min. Precipitate the product by adding cold diethylether (40 mL). Isolate the product by centrifugation at 1,000 × *g* (low brake speed) for 5 min at 4°C to afford H<sub>2</sub>N-Ahx<sub>3</sub>Leu<sub>3</sub>VS **12** as a white solid (10.6 mg, 14 μmol) in >98% yield.
6. Dissolve H<sub>2</sub>N-Ahx<sub>3</sub>Leu<sub>3</sub>VS **12** (10.6 mg, 14 μmol) in DMF (1 mL) and add Me<sub>4</sub>BodipyFL-NHS ester **7** (6.4 mg, 15.5 μmol) and DIPEA (24 μL, 0.14 mmol) (see Note 6). Stir the resulting solution at room temperature under an argon atmosphere for 16 h. Concentrate the solution in vacuo to dryness and purify by reversed-phase HPLC to obtain the fluorescent proteasome probe Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS (**6** mg, 5.7 μmol) in 40% yield (see Fig. 5 for LC/MS data). Fluorescence data: λ<sub>ex</sub> = 515 nm, λ<sub>em</sub> = 519 nm.

### **3.2. Profiling of Proteasome Activity Using SDS-PAGE Based Assays**

#### *3.2.1. In Vitro Profiling of Proteasome Activity in Cell Lysates*

1. Grow the cell line of choice at 37°C and 5% CO<sub>2</sub> in a humidified incubator in the appropriate medium supplemented with 10% FCS until log-phase (suspension cells) or until 80% confluency is reached (adherent cells). Medium may be supplemented with antibiotics.
2. Harvest suspension cells by centrifugation. To this end, transfer cells to a Falcon tube and centrifuge at 1,200 × *g* for 2 min at 4°C (see Note 7). Discard the supernatant and resuspend the cell pellet in 10–20 pellet volumes of PBS. Transfer cells to an Eppendorf tube and pellet cells by centrifugation at 1,200 × *g* for 2 min at 4°C. Discard the supernatant. Harvest adherent cells by trypsinization. To this end, aspirate the medium, wash cells with PBS and aspirate. Add just enough trypsin to cover the cells. As soon as cells detach, add medium containing 10% FCS to the cells to inactivate the trypsin and transfer the cells to a Falcon tube. Pellet cells by centrifugation at 1,200 × *g* for 2 min at 4°C. Discard the supernatant and resuspend the cell pellet in 10–20 pellet volumes of PBS. Transfer cells to an Eppendorf tube and pellet cells by centrifugation at 1,200 × *g* for 2 min at 4°C. Discard the supernatant.
3. Resuspend cells in 2 pellet volumes of cold HR buffer.
4. Lyse cells mechanically (e.g., by sonication using the Bioruptor according to the manufacturer's instructions). **Critical:** For proper labeling, do not add detergent to the lysis buffer. If detergent is absolutely required for proper cell lysis, keep the detergent concentration as low as possible.
5. Centrifuge for at 14,000 × *g* for 3 min at 4°C to remove membrane fractions and cell debris and transfer the supernatant to a fresh Eppendorf tube.
6. Determine protein concentrations using a Bradford assay. For incubation with proteasome inhibitors prior to proteasome labeling, proceed to step 7. To label the proteasome directly, proceed to step 9. **Critical:** Proceed with labeling directly and do not store nonincubated lysates. The quality of labeling will decrease if lysates are freeze–thawed.

*Option 1:* Incubation with proteasome inhibitors followed by labeling with probe.

7. To study the effects of proteasome inhibitors, transfer 25 µg of lysate to a fresh Eppendorf tube and adjust the volume to 24.5 µL with HR buffer. Add 0.5 µL of a 50× stock solution of the desired proteasome inhibitor in DMSO to obtain the desired 1× concentration of proteasome inhibitor and a final protein concentration of 1 µg/µL. Include a reference sample to which 0.5 µL DMSO but no proteasome inhibitor is added. Vortex and incubate the samples for the desired time period at 37°C.

8. Add 0.5  $\mu\text{L}$  of a 50  $\mu\text{M}$   $\text{Me}_4\text{BodipyFL-Ahx}_3\text{Leu}_3\text{VS}$  stock solution in DMSO to the samples to obtain a final concentration of 1  $\mu\text{M}$   $\text{Me}_4\text{BodipyFL-Ahx}_3\text{Leu}_3\text{VS}$ . Vortex and incubate for 1 h at 37°C. Proceed to Subheading 3.2.4.

**Pause point:** At this point, incubated lysates can be snap-frozen in liquid  $\text{N}_2$  and stored at  $-20^\circ\text{C}$  until further use.

*Option 2:* Incubation with probe only.

9. To label proteasome subunits directly, transfer 25  $\mu\text{g}$  lysate to a fresh Eppendorf tube and adjust the volume to 24.5  $\mu\text{L}$  with HR buffer. Add 0.5  $\mu\text{L}$  of a 50  $\mu\text{M}$   $\text{Me}_4\text{BodipyFL-Ahx}_3\text{Leu}_3\text{VS}$  stock solution in DMSO to obtain a final probe concentration of 1  $\mu\text{M}$  and a final protein concentration of 1  $\mu\text{g}/\mu\text{L}$ . Vortex and incubate the sample for 1 h at 37°C. Proceed to Subheading 3.2.4.

**Pause point:** At this point, incubated lysates can be snap-frozen in liquid  $\text{N}_2$  and stored at  $-20^\circ\text{C}$  until further use.

### 3.2.2. Labeling of Active Proteasome Subunits in Living Cells

Labeling of active proteasome subunits can be performed in suspension cells (proceed to step 1) or adherent cells (proceed to step 5).

#### Suspension cells

1. Grow the cell line of choice in appropriate medium containing 10% FCS at 37°C and 5%  $\text{CO}_2$  in a humidified incubator until log-phase is reached. Medium may be supplemented with antibiotics.
2. Count cells, transfer cells to a Falcon tube, and pellet cells by spinning at  $1,200\times g$  for 2 min at room temperature. Discard the supernatant.
3. Resuspend cells in fresh medium at  $0.5\times 10^6$ – $1.0\times 10^6$  cells/mL.
4. Add 0.5 mL of cell suspension ( $0.25\times 10^6$ – $0.5\times 10^6$  cells) to each well of a 24-well plate (or as many wells as needed). For incubation with proteasome inhibitors prior to proteasome labeling, proceed to step 7. To label proteasome only, directly proceed to step 8.

#### Adherent cells

5. Grow the cell line of choice in a 24-well plate in appropriate medium supplemented with 10% FCS at 37°C and 5%  $\text{CO}_2$  in a humidified incubator until 80% confluency is reached. Medium may be supplemented with antibiotics.
6. Aspirate the medium and add 0.5 mL of fresh medium to the cells. For incubation with proteasome inhibitors prior to proteasome labeling, proceed to step 7. To label proteasome only, directly proceed to step 8.



7. To study the effects of proteasome inhibitors, add 1  $\mu\text{L}$  of a 500 $\times$  stock solution of the desired proteasome inhibitor in DMSO to each well to obtain the desired 1 $\times$  concentration of proteasome inhibitor. Include a reference sample to which 1  $\mu\text{L}$  DMSO but no proteasome inhibitor is added. Incubate the cells for the desired time period in an incubator.
8. Add 5  $\mu\text{L}$  of a 50  $\mu\text{M}$  Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS stock solution in DMSO to each well to obtain a final probe concentration of 500 nM. Incubate the cells for 1 h in an incubator. If cells do not tolerate 1% DMSO, 1  $\mu\text{L}$  of a 250  $\mu\text{M}$  Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS stock solution in DMSO can be added alternatively.
9. To block all remaining proteasome activity and prevent postlysis labeling events, add 1  $\mu\text{L}$  of a 5 mM MG132 stock solution in DMSO to each well to obtain a final MG132 concentration of 10  $\mu\text{M}$ . Incubate the cells for 1 h in an incubator. Step 9 can be omitted if no differences in labeling are observed between samples that are incubated with MG132 before cell harvest and samples that are harvested directly after step 8.
10. To harvest suspension cells: transfer the cells in each well to an Eppendorf tube and pellet cells by centrifugation at 1,200  $\times g$  for 2 min at 4°C. Discard the supernatant and resuspend the cell pellet in 1 mL PBS. Pellet cells by centrifugation at 1,200  $\times g$  for 2 min at 4°C. Discard the supernatant. To harvest adherent cells: aspirate the medium, wash the cells by adding 1 mL of PBS to each well, and aspirating the PBS. Add 50  $\mu\text{L}$  trypsin to each well. As soon as cells detach, add 1 mL of fresh medium containing 10% FCS to the cells to inactivate the trypsin. Transfer the cells in each well to an Eppendorf tube and pellet cells by centrifugation at 1,200  $\times g$  for 2 min at 4°C. Discard the supernatant and resuspend the cell pellet in 1 mL PBS (see Note 7). Pellet the cells by centrifugation at 1,200  $\times g$  for 2 min at 4°C. Discard the supernatant.

**Pause point:** At this point, incubated cell pellets can be snap-frozen in liquid N<sub>2</sub> and stored at -20°C until further use.

11. Resuspend cells in 1–2 pellet volumes of cold NP40 lysis buffer and lyse for 30 min at 4°C (see Note 7).
12. Centrifuge at 14,000  $\times g$  for 3 min at 4°C to remove membrane fractions and cell debris. Transfer the supernatant to fresh Eppendorf tube.
13. Determine protein concentrations using a Bradford assay.
14. Transfer 25  $\mu\text{g}$  lysate to a fresh Eppendorf tube and adjust the final volume to 25  $\mu\text{L}$  with NP40 lysis buffer to obtain a final protein concentration of 1  $\mu\text{g}/\mu\text{L}$ . Proceed to Subheading 3.2.4.

**Pause point:** At this point, cell lysates can be snap-frozen in liquid N<sub>2</sub> and stored at -80°C until further use.

3.2.3. *Ex Vivo Profiling of Proteasome Subunit Activity in Murine Tissues*

1. Remove the tissue types to be analyzed and rinse with PBS.
2. Grind the tissue, transfer to an Eppendorf tube, and add 1–2 volumes of cold HR buffer.
3. Add 1 volume of glass beads ( $\leq 106 \mu\text{m}$ , acid washed, Sigma) to the tissue. Lyse cells mechanically by vortexing at high speed for 45 min at  $4^\circ\text{C}$  (see Note 7). **Critical:** For proper labeling, do not add detergent to the lysis buffer. If detergent is absolutely required for proper cell lysis, keep the detergent concentration as low as possible.
4. Remove beads, membrane fractions, and cell debris by centrifugation at  $14,000\times g$  for 5 min at  $4^\circ\text{C}$  and transfer the supernatant to a fresh Eppendorf tube.
5. Determine protein concentrations using a Bradford assay. **Critical:** Proceed with labeling directly and do not store non-incubated lysates. The quality of labeling will decrease if lysates are freeze–thawed.
6. Transfer  $25 \mu\text{g}$  lysate to a fresh Eppendorf tube and adjust the volume to  $24.5 \mu\text{L}$  with HR buffer. Add  $0.5 \mu\text{L}$  of a  $50 \mu\text{M}$   $\text{Me}_4\text{BodipyFL-Ahx}_3\text{Leu}_3\text{VS}$  stock solution in DMSO to obtain a final probe concentration of  $1 \mu\text{M}$  and a final protein concentration of  $1 \mu\text{g}/\mu\text{L}$ . Vortex and incubate the samples for 1 h at  $37^\circ\text{C}$ . Proceed to Subheading 3.2.4.

**Pause point:** At this point, incubated lysates can be snap-frozen in liquid  $\text{N}_2$  and stored at  $-20^\circ\text{C}$  until further use.

3.2.4. *Gel Electrophoresis and In-Gel Fluorescence Readout*

1. These instructions assume the use of the NuPAGE precast gel system (Invitrogen) and precast mini gels to separate proteins on SDS-PAGE. If a mini gel does not separate the individual proteasome subunits sufficiently, a larger gel system can be used, e.g., the PROTEAN II xi Cell system from Biorad ( $16\times 20$  cm glass plates, 12.5% separating gel, 4% stacking gel.) When using this system, increase the sample volume to  $50 \mu\text{L}$  per sample. Do not change the final concentrations of proteasome inhibitors, protein, and probe in the samples. Load  $20\text{--}30 \mu\text{L}$  sample per well and run at 10 mA for 16 h. Subsequently, increase the current to 35 mA, wait until the blue front has run off the gel and run for another hour before removing the gel from the system. The protocol described below can also be adapted to other gel systems. Use common protocols for denatured samples. Separate proteins using a 12 or 12.5% separating gel and a 4% stacking gel.
2. Add  $12.5 \mu\text{L}$   $3\times$  reducing sample buffer (see Subheading 2.2.4 for buffer recipe) to each sample obtained in Subheadings 3.2.1–3.2.3. The volume of these samples should be  $25 \mu\text{L}$ . Vortex and denature by boiling the sample for 10 min at  $70^\circ\text{C}$ . Centrifuge at  $14,000\times g$  for 1 min at room temperature.

Denatured samples can be stored at  $-20^{\circ}\text{C}$  for later use. **Critical:** When using the NuPAGE gel system also use the NuPAGE LDS Sample buffer to prepare the  $3\times$  reducing sample buffer. The use of a different reducing sample buffer leads to improper running of the gel, resulting in fuzzy and unfocused bands.

3. Assemble the NuPAGE gel unit using a precast NuPAGE 12% Bis-Tris gel according to the manufacturer's instructions. Add  $1\times$  MOPS buffer to both the inner and outer chamber of the gel unit. Add  $125\ \mu\text{L}$  antioxidant to the inner gel chamber only. Load  $10\ \mu\text{L}$  of denatured sample per well. Keep one well free and load this well with  $6.5\ \mu\text{L}$  prestained molecular weight marker (e.g., SeeBlue<sup>®</sup> Plus2 Pre-Stained Standard from Invitrogen). Load  $3\ \mu\text{L}$  of  $3\times$  reducing sample buffer to any remaining wells.
4. Run the gel at 170–180 V. **Critical:** For proper separation of the  $\beta 1\text{i}$  and  $\beta 5$  subunits, run the gel until the 15 kDa protein in the molecular weight marker (Lysozyme in the SeeBlue<sup>®</sup> Plus2 Pre-Stained Standard) is at the bottom of the gel.
5. Remove the gel from the cassette and image the wet gel slab for 10–120 s using a fluorescence imager containing an appropriate filter set (excitation at 480 nm, emission at 530 nm).
6. Analyze images using appropriate software to quantify fluorescence intensities.

### 3.3. High-Throughput FACS-Based Proteasome Activity Assay

1. Seed MeJuso cells in a 96-well flat bottom tissue culture plate in DMEM supplemented with 10% FCS and antibiotics. Add 10,000 cells in a total volume of  $100\ \mu\text{L}$  per well (see Note 8).
2. Place cells at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in a humidified incubator and let cells attach for 16–24 h.
3. To screen compounds for their effects on proteasome activity, add  $1\ \mu\text{L}$  of 2.5 mM stock solutions of the desired compounds in DMSO to  $99\ \mu\text{L}$  medium to obtain  $5\times$  compound solutions. To obtain  $5\times$  control solutions, add  $1\ \mu\text{L}$  of a 0.4 mM stock solution of MG132 in DMSO (positive control, 100% inhibition) or  $1\ \mu\text{L}$  DMSO only (negative control, 0% inhibition) to  $99\ \mu\text{L}$  medium.
4. Remove medium from the cells, add  $80\ \mu\text{L}$  of fresh medium to each well and  $20\ \mu\text{L}$  of the  $5\times$  solutions of compounds and controls obtained in step 3 (then the final concentration of compound in each well is  $5\ \mu\text{M}$ , final concentration of MG132 is 800 nM). Incubate cells for 16 h in an incubator.
5. Make a  $42\times$  dilution in DMEM of a  $50\ \mu\text{M}$   $\text{Me}_4\text{BodipyFL-Ahx}_3\text{Leu}_3\text{VS}$  DMSO stock solution to obtain a  $1.2\ \mu\text{M}$  probe solution (e.g., for one 96-well plate, add  $48\ \mu\text{L}$  of a  $50\ \mu\text{M}$

Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS DMSO stock solution to 1,952 μL DMEM). Add 20 μL of the 1.2 μM probe solution to each well to obtain a final probe concentration of 200 nM. Incubate cells for 2 h in an incubator.

6. Discard the supernatant, add 200 μL PBS to each well to wash the cells, and discard the PBS.
7. Harvest cells. Add 20 μL trypsin to each well. As soon as cells detach, add 55 μL FACS buffer and 25 μL fixation buffer to each well to obtain a final formaldehyde concentration of 0.5% (see Subheading 2.3 for buffer recipes). Fix cells by shaking the plate on a shaker for at least 20 min.

**Pause point:** At this point, fixed cells can be stored at 4°C until further use.

8. Measure intracellular fluorescence in the cells by flow cytometry. Fluorescence is measured in the F11 channel (530/30 filter).

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## 4. Notes

1. All buffers are prepared in water that has a resistivity of 18.2 MΩ cm (MilliQ water).
2. Store stock solutions of ATP and DTT in water in aliquots at -20°C.
3. A shorter synthetic route to the synthesis of Me<sub>4</sub>BodipyFL-Ahx<sub>3</sub>Leu<sub>3</sub>VS involves the use of a hyper acid-labile resin and introduction of the Me<sub>4</sub>BodipyFL fluorophore on solid phase. This route, however, requires larger amounts of fluorophore NHS ester. Synthesize the peptide as described in step 1, starting from preloaded hyper acid-labile resin. After the final coupling step, remove the Fmoc group and wash the resin thoroughly. Subsequently, resuspend the resin in NMP (10 mL/g of resin) and add five equivalents of Me<sub>4</sub>BodipyFL-NHS ester and five equivalents of DIPEA and allow the reaction to proceed for 16 h. Wash the resin thoroughly and cleave the peptide from the resin using 2% TFA in CH<sub>2</sub>Cl<sub>2</sub> or 25% HFIP (or according to protocol of the manufacturer of the hyper acid-labile resin) in CH<sub>2</sub>Cl<sub>2</sub> and allow the cleavage to take place for 20 min. Precipitate the product by adding 10 volume equivalents of cold diethylether/pentane (3:1 v/v) and isolate by centrifugation for 5 min at 1,000 ×g (low brake speed). Wash the pellet three times by adding cold diethylether/pentane (3:1 v/v) and centrifuging at 800 ×g (high brake speed). Continue with step 4 to obtain the final probe. Steps 5 and 6 are omitted in this procedure.

4. The use of low capacity and preloaded PEG polystyrene resin is recommended.
5. To circumvent the introduction of a Boc group on the N-terminus in solution as described in step 3, it is also possible to use Boc-6-aminohexanoic acid (Boc-Ahx-OH) instead of Fmoc-Ahx-OH, as a building block in the last coupling cycle. Synthesize the peptide as described in step 1, starting from preloaded hyper acid-labile resin. Couple Boc-Ahx-OH to the resin in the final coupling step. Wash the resin thoroughly and cleave the peptide from the resin using 2% TFA in  $\text{CH}_2\text{Cl}_2$ . Concentrate the compound under reduced pressure. Purify the compound as described in step 3 and continue with step 4.
6. Other commercially available NHS-activated dyes may also be used here, circumventing the synthesis of the  $\text{Me}_4\text{BodipyFL-NHS}$  ester. We recommend the use of BodipyFL-NHS ester or BodipyTMR-NHS ester (Invitrogen) for optimal labeling results in both cell lysates and intact cells (14).
7. Cell lysates or samples containing protease activity were kept on ice unless indicated otherwise.
8. This protocol describes a high-throughput FACS-based proteasome activity assay in a 96-well format using MeJuso cells. The protocol can easily be adapted to 384-well or 24-well format by changing the amounts of cells and volumes described in Section 3.3. Do not change the final concentrations of compounds and probe. The protocol can be performed with other adherent cells and can be adapted to suspension cells. When using suspension cells, perform wash steps by centrifugation ( $1,200 \times g$  for 5 min, then discard the supernatant and resuspend cells).

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