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A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers

Andrew L. Niles [a,*], Richard A. Moravec [a], P. Eric Hesselberth [b], Michael A. Scurria [b], William J. Daily [b], Terry L. Riss [a]

[a] *Promega Corp., Madison, WI 53711, USA*
[b] *Promega Bioscience, San Luis Obispo, CA 93401, USA*

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Abstract

A method to simultaneously determine the relative numbers of live and dead cells in culture by introducing a combination of two fluorogenic substrates or a fluorogenic and a luminogenic protease substrate into the sample is described. The method is based on detection of differential ubiquitous proteolytic activities associated with intact viable cells and cells that have lost membrane integrity. A cell-permeable peptide aminofluorocoumarin substrate detects protease activity restricted to intact viable cells. Upon cell death, the viable cell protease marker becomes inactive. An impermeable peptide rhodamine 110 (or aminoluciferin) conjugated substrate detects protease activity from nonviable cells that have lost membrane integrity. The multiplex assay can detect 200 dead cells in a population of 10,000 viable cells. The protease substrate reagents do not damage viable cells over the course of the assay, thus the method can be multiplexed further with other assays in a homogeneous format. Ratiometric measurement of viable and dead cells in the same sample provides an internal control that can be used to normalize data from other cell-based assays.

Keywords: Cell-based; Cytotoxicity; Viability; Multiplex; Fluorescence; Luminescence; Protease; Apoptosis; Homogeneous; High throughput

It is an important and necessary experimental practice to determine the viability of cells in culture after chemical, biological, or physical treatment and manipulation. Maintenance of membrane integrity is a common criterion for cell viability. Measurable changes in membrane permeability include trypan blue exclusion, nucleic acid staining, and ^{51}Cr or lactate dehydrogenase release [1,2]. Conversely, measures of viability by metabolic capacity include tritiated thymidine incorporation, ATP content, tetrazolium dye reduction, and fluorescein diacetate labeling [3].

These existing techniques have a number of technical or practical drawbacks which limit their utility in multiplexed or high-throughput formats. For instance, cellular ^{51}Cr release assays require significant prelabeling preparation, and all assays utilizing radiological tracers or mutagenic/teratogenic dyes impose significant exposure, handling, and disposal issues. In addition, tetrazolium or resazurin chemistries can significantly complicate additional downstream applications by color quenching of fluorescence or luminescence.

Cultured mammalian cells contain a rich milieu of protease, esterase, lipase, and nuclease activities which contribute to homeostatic maintenance. In particular, cytosolic, lysosomal, and transmembrane-bound proteases are involved in intracellular protein degradation, generation of immunogenic peptides, posttranslational modification, and cell division [4–6]. The activity of these enzymes is regulated by various mechanisms including specialized compartmentalization [7]. In response to extreme stress, environmental adversity, or committed progression of the apoptotic program, a loss of subcellular structure or membrane integrity is observed [8,9]. Therefore, we hypothesized that the release of stable protease activity into the

* Corresponding author. Fax: +1 608 298 4818.
E-mail address: andrew.niles@promega.com (A.L. Niles).

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cell culture medium in in vitro cell models could represent a potential enzymatic marker for cell death. Conversely, cytoenzymological staining of constitutive proteolytic enzymes could serve as a correlate of cellular viability. Together, these different proteolytic activities could allow for the simultaneous measurement of the relative number of viable or dead cells in a cell culture population.

Materials and methods

Cell lines

Jurkat, U266, HL-60, HeLa, HCT-116, MCF-7, PA-1, ACHN, PC-3, DU-145, H226, LN-18, HEK 293, HepG2, NK-92CI, U937, and SK-MEL-28 were obtained from American Type Culture Collection and maintained in respective growth media as recommended. Cells were counted and determined to exhibit viability of at least 95% by trypan blue exclusion prior to experimentation.

Cytotoxic agents and detergents

Anti-Fas mAb (clone CH-11) was obtained from Medical Biologic Laboratories. Staurosporine, ionomycin, and saponin were obtained from Sigma.

Cell-based protease assays

Ala-Ala-Phe-AMC was obtained from Promega. Z-Leu-Leu-Val-Tyr-aminoluciferin, Z-Leu-Arg-aminoluciferin, Z-Phe-Arg-aminoluciferin, Ala-Ala-Phe-aminoluciferin, bis-Ala-Ala-Phe-R110 (bis-AAF-R110), and bis-Gly-Phe-R110 were synthesized by Promega Biosciences. Suc-Ala-Ala-Phe-AMC, H-Phe-AMC, H-Tyr-AMC, Glutyl-Ala-Ala-Phe-AMC, H-Gly-Phe-AMC, Z-Gly-Ala-Met-AMC, Suc-Leu-Leu-Val-Tyr-AMC, D-Ala-Leu-Lys-AMC, H-Gly-Ala-AMC, H-Gly-Gly-AMC, Suc-Ala-Ala-Phe-AMC, Z-Arg-Leu-Arg-Gly-Gly-AMC, Z-Leu-Arg-Gly-Gly-AMC, and Ac-Ala-Ala-Tyr-AMC were sourced from Bachem. Gly-Phe-AFC (GF-AFC), Pro-Phe-Arg-AMC, Gly-Gly-Leu-AMC, and Ser-Tyr-AFC were obtained from Calbiochem. Z-Phe-Arg-AMC and Suc-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-AMC were purchased from Sigma. All substrates were solubilized with dimethyl sulfoxide (DMSO)[1] from 10 to 100 mM depending on solubility.

HL-60, HeLa, and Jurkat cells were used for the differential protease activity screen. Portions of the cells were treated by freeze fracture, mild sonication, or detergent lysis to simulate cytotoxicity. Lysis by sonication was achieved using a microtip Misonix 3000 with 30% power for three 5-pulses. Disruption of membrane integrity was confirmed for all methods by microscopic analysis using trypan blue staining. An untreated aliquot of matched cells served as the viability control.

Fluorogenic substrates were diluted from 100 μM to 1 mM in 10 mM Hepes, pH 7.5, or recommended cell culture medium with 10% serum. Luminogenic substrates were suspended to 500 μM in a luciferin detection reagent (Promega V859A) and resuspended in 100 mM Hepes, pH 7.5. The substrate reagents were then introduced in 1/10th or 1/5th volumes, respectively, into samples of cultured cells and incubated at 37 °C in a Me'Cour circulating heat block controlled by a Caron 2050W exchange unit for 30 min. The resulting fluorescence or luminescence was measured using a CytoFluor™ II fluorometer or BMG-PolarStar™ multimode fluorometer/luminometer.

Differential protease activity inhibition/augmentation profiles

Puromycin, E-64, phenylmethylsulfonyl fluoride, adenosine 5'-triphosphate (ATP), *N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp disodium salt (phosphoramidon), *N*-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutyryl-L-leucine hydrochloride (bestatin), 1,10-phenathroline, 3,4-diisocoumarin, 4-(2-aminoethyl)benzenesulfonyl fluoride, 1,4-dithio-DL-threitol (DTT), edetate disodium dihyrdrate (EDTA), isovaleryl-L-valyl-L-valyl-[(3S,4S)-4-amino-3-hydroxy-6-methylheptanoyl]-L-alanyl[93S,4S]-4-amino-3-hydroxy-6-methylheptanoicacid (pepstatin A), sodium chloride, and protinin, N-acetyl-L-leucyl-L-leucyl-L-argininal hemisulfate salt (leupeptin) were purchased from Sigma. Protease inhibitors were resuspended in DMSO to varying stock concentrations with a high target concentration of 200 μM or 200 μg/ml in Dulbecco's phosphate buffer saline w/o Mg^{2+} or Ca^{2+} (DPBS) for addition to either lysates or viable cell populations. DTT, NaCl, EDTA, and ATP were also diluted in DPBS. All compounds were incubated with nonviable or viable cells for 30–60 min at 37 °C prior to assessment of activity.

The viable cell protease marker assays were conducted with mixtures of sonicated or saponin-lysed and viable HL-60 cells using GF-AFC at 100 μM final concentration. The nonviable cell protease release assays were conducted with mixtures of sonicated or saponin-lysed and viable HL-60 and/or SK-MEL-28 cells using Ala-Ala-Phe-AMC or bis-AAF-R110 at final concentration of 50 μM.

Enzyme activity half-life comparison

HL-60 cells were seeded into 96-well plates at a density of 10,000 cell per well in 100 μl medium. Saponin (5 μl; 0.2% final concentration) was added to replicate sets of wells at 1-h intervals over a 7-h time course and mixed briefly by orbital shaking to lyse cells and release cytoplasmic contents into the medium. During this same time frame, an equal volume of RPMI 1640 with 10% FBS was added to viability control wells. Protease activity released from dead cells was measured by adding the luminogenic AAF-aminoluciferin

[1] *Abbreviations used:* DMSO, dimethyl sulfoxide; DTT, dithiothreitol; DPBS, Dulbecco's phosphate buffer saline; FBS, fetal bovine serum; AK, adenylate kinase; LDN, lactate dehydrogenase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

protease detection reagent as described above. Adenylate kinase (AK) activity was measured in parallel wells using the ToxiLight® Non-Destructive Cytotoxicity BioAssay Kit as recommended by the manufacturer (Cambrex). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) activity was measured using the aCella™-Tox kit as per manufacturer's directions (Cell Technology, Inc). Data were recorded using a BMG PolarStar™.

Correlation with conventional viability and cytotoxicity assays

Jurkat cells were adjusted to 100,000 cells/ml and divided into two pools. One pool was sonicated, the other was left untreated. The two pools were blended at various ratios to represent viability from 0 to 100%. The samples were then added to replicate wells of a 96-well plate in 100 μl volumes. GF-AFC or bis-AAF-R110 protease substrates were diluted to 100 μM in 100 mM Hepes, pH 7.5, and 100 μl of the appropriate substrate was added to the samples. Other chemistries for the measurement of cytotoxicity and viability were added to parallel plates. The CytoTox-ONE™ Cytotoxicity Assay (Promega) was used to measure the released lactate dehydrogenase (LDH). Ethidium homodimer (Invitrogen) was used to stain genomic DNA from cells with compromised membrane integrity. The CellTiterBlue™ Assay (Promega) was used to measure resazurin reduction to resorufin as a marker of viable cells and the CellTiter-Glo™ Assay (Promega) was used to measure ATP as a marker of viable cells.

Multiplexed cytotoxicity assays

Staurosporine was twofold serially diluted in RPMI 1640 + 10% FBS in a 96 well culture plate in replicate 50-μl volumes. Medium without staurosporine was added to replicate wells to serve as untreated cell and medium-only controls. Jurkat cells were seeded at a density of 5000 cells per well in 50 μl to all wells, except control medium replicates which received an additional volume of medium. The plate was mixed briefly by orbital shaking and incubated for 48 h at 37 °C in 5% CO_2. Gly-Phe-AFC substrate was diluted to 200 μM in 100 mM Hepes with 50 mM $MgSO_4$, pH 7.5 and added in 50 μl volumes to all wells. After 30 min of incubation at 37 °C, the resulting fluorescence was measured in the AFC (400_{Ex} 505_{Em}) channel using a BMG Fluostar Optima. Ala-Ala-Phe-aminoluciferin was made 200 μM in 5 ml of the above Hepes buffer with $MgSO_4$ and used to rehydrate a luciferin detection reagent vial; 50 μl of the solution was added to all wells, and the plate was incubated for an additional 15 min at room temperature prior to measurement of luminescence using a BMG Fluostar Optima.

Ionomycin was twofold serially diluted in the same manner as that described for staurosporine, with the same assay controls. Jurkat cells were seeded at a density of 10,000 cells/well in 50 μl to all wells, except control medium replicates which received an additional volume of medium. The plate was mixed briefly by orbital shaking and incubated for 6 h at 37 °C in 5% CO_2. GF-AFC and bis-AAF-R110 were added to 100 mM Hepes, pH 7.5, to a final concentration of 100 μM. 100 μl of this solution was added to all wells. After 30 min of incubation at 37 °C, the resulting fluorescence was measured in the AFC (400_{Ex} 505_{Em}) and R110 (485_{Ex} 520_{Em}) channels using a BMG Fluostar Optima.

Multiplexed cytotoxicity and caspase assays

Anti-Fas mAb was twofold serially diluted in RPMI 1640 + 10% FBS in opaque 96-well plates in 50 μl volumes. Medium alone served as control. Jurkat cells were seeded at 10,000 cells/well in 50 μl and the plates incubated at 37 °C in 5% CO_2 for 8 h. A fluorogenic viability and cytotoxicity reagent was prepared by adding GF-AFC and bis-AAF-R110 substrates to 1 mM in 100 mM Hepes, pH 7.5; 10 μl of the reagent was delivered to all wells, mixed by orbital shaking, and returned to a 37 °C incubator for 30 min of incubation. The resulting fluorescence was measured in the AFC and R110 channels as described previously. Caspase-Glo® 3/7 reagent (Promega) was prepared as directed and added in equal volumes to the well. Luminescence was measured as described previously.

Results

Differential protease activity screen

A variety of substrates were screened to identify substrate preferences for proteases released from compromised cells and those retained in viable cells (Table 1). Peptide substrates blocked on the N terminus with benzyloxycarbonyl or succinylated or acetylated amino-terminal groups were tested to determine whether an endo- or an exopeptidase activity predominated. Substrates with a free amino terminus were examined to detect the contribution of aminopeptidase activities or endoproteolytic activities sterically hindered by bulky blocking groups. From this panel, at least three proteolytic profiles emerged: an aminopeptidase-like activity preferring unblocked Ala-Ala-Phe tripeptide, a proteosomal (chymotrypsin-like) activity measured by release of blocked Leu-Leu-Val-Tyr peptides, and a labile activity acting on Gly-Phe, Gly-Ala, Phe-, Tyr-, or Gly-Gly-Leu substrates. (Table 1). The latter activities were largely restricted to intact viable cells. The substrates that selectively labeled live cells contained unblocked mono-, bi-, or tri-peptide sequences which can enter the cytoplasm of viable cells. Fig. 1 shows that viable cells incubated with the permeable GF-AFC substrate generated a substantially greater fluorescent signal than an equivalent number of cells that were treated by sonication to disrupt the cell membrane. The fluorescent signal resulting from incubation with GF-AFC was directly proportional to the number of viable cells. Disruption of the cell

Table 1
Proteolytic substrates tested for differential activity in cells

Substrate	Known protease(s)	Viability	Cytotoxicity
Z-FR-AMC	Cathepsin B, L	None[a]	None
Z-GGL-AMC	20S proteosome	++	None
Z-LLRGG-AMC	Isopeptidase T	None	None
Z-LRGG-AMC	Isopeptidase T	None	None
SRPFHLLVY-AMC	Proteosome	None	None
PFR-AMC	Kallikrein	None	None
GG-AMC	Aminopeptidase	None	None
GA-AMC	Aminopeptidase	++	None
ALK-AMC	Plasmin	None	None
AAF-AMC	Tripeptidyl peptidase II	None	+++++
bis-AAF-R110	Tripeptidyl peptidase II	None	+++++
AAF-aminoluciferin	Tripeptidyl peptidase II	None	+++++
Glutyl-AAF-AMC	Chymotrypsin	None	None
GF-AFC	Cathepsin C	+++++	None
GF-AMC	Cathepsin C	++	None
bis-GF-R110	Cathepsin C	None	None
Suc-LLVY-AMC	Calpain	None	+
Suc-LLVY-aminoluciferin	Calpain	None	++
Suc-AAF-AMC	Chymotrypsin	None	None
Ac-AAY-AMC	Chymotrypsin	None	None
Z-LR-aminoluciferin	Cathepsin K	None	None
Z-FR-aminoluciferin	Cathepsin B, L	None	None
SY-AFC	Aminopeptidase	None	None
F-AMC	Aminopeptidase M	+++	None
Y-AMC	Cathepsin H	++	None

[a] None denotes no statistical activity above control population. Scoring based on practical sensitivity. Practical sensitivity is calculated as Net Fluorescence (treated-untreated or untreated-treated) /standard deviation of control. This value must be greater than 3 as per Zhang et al. [15]. (+) >2500 cells/well; (++) >2000 cells/well; (+++) >1000 cells/well; (++++) >200 cells/well; (+++++) <200 cells/well.

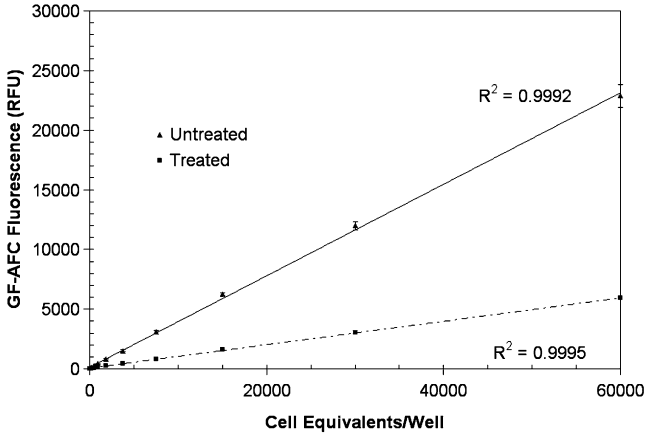

Fig. 1. Differential signal response of GF-AFC in the presence of treated and untreated equivalents of U266 cells.

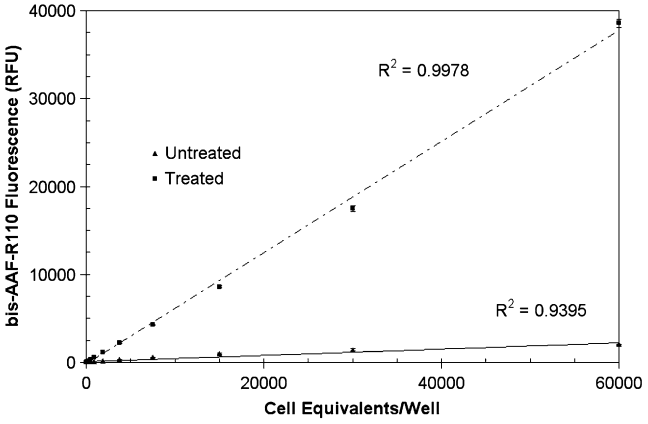

Fig. 2. Differential signal response of bis-AAF-R110 in the presence of treated and untreated equivalents of U266 cells.

membrane by sonication (or detergent treatment, not shown) resulted in a diminished ability to generate a signal from the GF-AFC substrate, suggesting a loss of proteolytic activity following cell disruption. It is interesting to note that bis-GF-R110 was not substantially cleaved by viable cell populations, possibly because of the inability to permeate the cell membrane.

Fig. 2 demonstrates that a viable cell population incubated with bis-AAF-R110 generated a signal only slightly above that of background fluorescence, whereas an equivalent number of cells that had been disrupted by sonication generated a substantially larger signal (15-fold increase with 60,000 cells). The protease activity that cleaved the bis-AAF-R110 substrate remained active after cell disruption. The selective detection of viable cells at 505 nm using the GF-AFC substrate and selective detection of disrupted cells at 527 nm using the bis-AAF-R110 substrate enabled development of a method for the simultaneous quantitative detection of live and dead cells in the same sample. The protease activity released from disrupted cells generated a strong signal after incubation with AAF-AMC, bis-AAF-

R110, or AAF-aminoluciferin substrates (Table 1). The use of different fluorophores or aminoluciferin as the leaving group provided greater flexibility for the choice of indicators used for multiplex detection of viable cells.

Characterization of protease activity profiles

We investigated the properties of the protease activities associated with viable and dead cells by treating cell preparations with various protease inhibitors or adjuncts shown in Table 2. Treatment of viable cells with puromycin (100 μg/ml), EDTA (10 mM), or bestatin (10 μM) inhibited generation of fluorescent signal from the GF-AFC substrate. This activity is ATP and DTT independent (no restoration of activity with 2 and 10 mM, respectively) and insensitive to halides (Cl^- supplied as 100 mM NaCl). The pattern of inhibition in viable cells is consistent with a serine or cysteine protease activity being responsible for cleavage of GF-AFC. At least some of the inhibitor profile characteristics are consistent with aminopeptidase PS [5,10] and/or the neutral dipeptidyl aminopeptidase described by Doughty and Gruenstein [11].

Perhaps the most interesting feature of this activity, which has not been previously been described, is that the enzyme(s) responsible for the protease viability activity appear(s) to have an almost absolute requirement for cell viability and is only marginally detected outside of compromised cells. Furthermore, this requirement was demonstrated to be closely related to inherent ATP levels or resazurin reduction, both reliable indicators of cellular viability (Fig. 3).

The Ala-Ala-Phe-utilizing protease activity indicative of cytotoxicity was sensitive to some serine protease inhibitors but not those with selectivity for trypsin or chymotrypsin-like activities. Treatment with cysteine protease inhibitors indicated that there was no apparent requirement for thiols to maintain activity of the dead cell marker. Treatment with specific inhibitors of aspartic and metallo-proteases also did not reduce activity. The activity profile was consistent with the abundant and conserved cytosolic protease, tripeptidyl peptidase II [12–14].

Correlation and comparison with existing markers

The ability of viable cells to generate a fluorescent signal from GF-AFC was correlated with other markers of cell viability. Fig. 3A shows a strong correlation between a luminescent assay measuring ATP as an indicator of cell viability and the use of GF-AFC. Fig. 3B shows a similar strong correlation between fluorescent signals resulting from incubation of cells with either GF-AFC substrate or resazurin as markers of cell viability.

The dead cell protease marker correlated well with other assays used to detect cells that have lost membrane integrity. Fig. 4 illustrates a strong correlation between the fluorescent dead cell protease activity and a fluorescent assay

Table 2
Inhibition or augmentation profiles of viability and cytotoxicity markers

Inhibitor/adjunct	Target class	Effect	Comments
Viability protease marker			
Puromycin	Aminopeptidase	Inhibition	Modest
EDTA	Metallo	Inhibition	Reduces viability
DTT	Cysteine	Augments	Modest in lysate
NaCl	Aminopeptidase	None	Insensitivity
1,10 Phenanthroline	Metalloprotease	None	to 100 μM
Bestatin	Aminopeptidase	Inhibition	Strong
3,4 Diisocoumarin	Serine	Inhibition	Kills cells
Phosphoramidon	Metallo	None	to 100 μM
E-64	Cysteine	None	to 100 μM
PMSF	Serine/cysteine	None	to 100 μM
ATP	ATP dependent	None	to 100 μM
Cytotoxicity protease marker			
Bestatin	Metalloprotease	None	to 10 μM
EDTA	Metalloprotease	None	to 50 mM
Pepstatin	Aspartic	None	to 100 μM
AEBSF	Serine	Inhibition	Strong
PMSF	Serine/cysteine	Inhibition	Strong
Aprotinin	Serine	None	to 100 μM
Leupeptin	Serine	None	to 100 μg/ml
Antitrypsin	Serine	None	to 100 μg/ml
FPR-CMK	Serine/cysteine	Inhibition	Modest
DTT	Cysteine	Augments	Modest
3,4 Diisocoumarin	Serine	Inhibition	Strong
E-64	Cysteine	None	to 100 μM
1,10 Phenanthroline	Metalloprotease	None	to 100 μM
Phosphoramidon	Metalloprotease	None	to 100 μM

Strong, <10 μM; Modest, <100 μM; None, no effect.

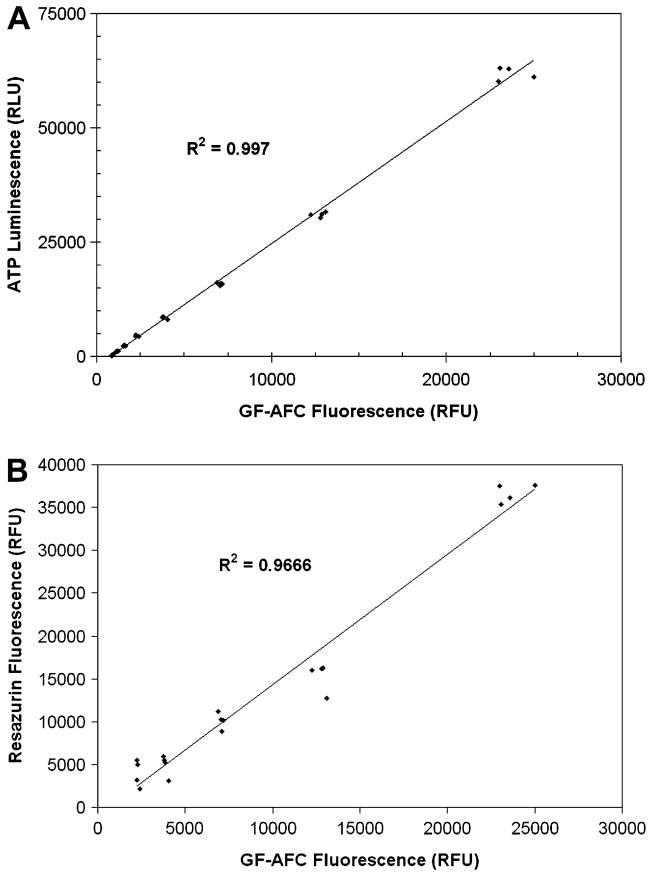

Fig. 3. Viability by protease activity is closely correlated with other measures of viability in treated cell populations. (A) GF-AFC activity vs ATP concentration. (B) GF-AFC activity vs metabolic resazurin reduction.

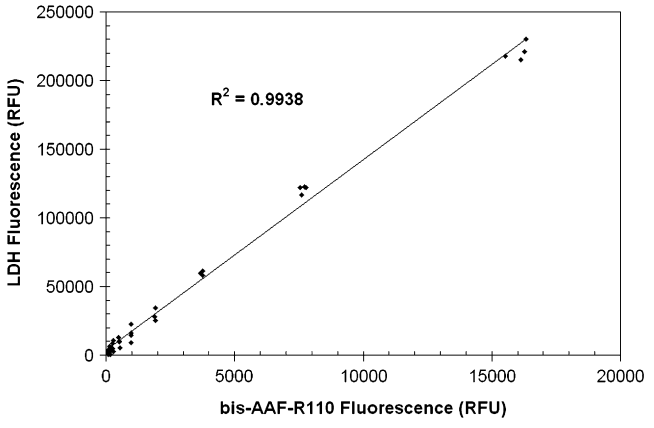

Fig. 4. Cytotoxicity by protease activity is closely correlated with other measures of cytotoxicity in treated cell populations. bis-AAF-R110 activity vs LDH activity.

measuring the release of lactate dehydrogenase. A similar strong correlation ($r^2 = 0.9979$) was demonstrated using ethidium homodimer staining of cells that have lost membrane integrity (not shown).

The half-life of the dead cell marker activity was compared to those of AK and G3PDH (Fig. 5). The half-life

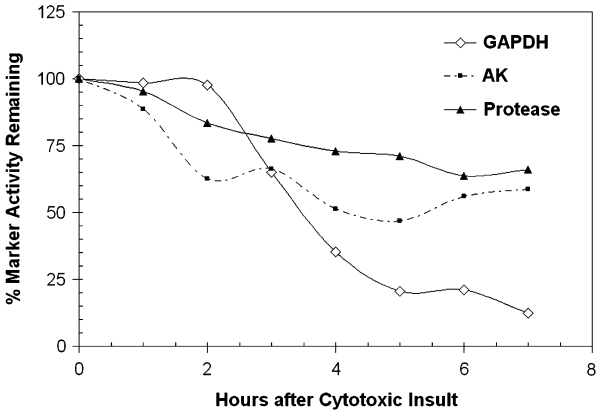

Fig. 5. Enzymatic surrogate marker half-life after cytotoxic insult. AK and G3PDH reagents prepared and added as directed by manufacturer. Luminescence measured after 5 min of reagent contact. Data are plotted as a function of maximal activity at zero time point.

of the dead cell marker activity was estimated to be 9–10 h suggesting that it was more stable than either AK or G3PDH when released into the culture medium. The stability of markers released into the culture medium is important to consider when interpreting data from cytotoxicity assays. If toxin-treated cells die within the first few hours of a 24 h incubation period, the changing activity of the marker must be considered to avoid underestimating the toxic response. Control samples to determine the total marker activity at the beginning and end of the incubation period can aide data interpretation.

Protease assay sensitivities

The assay sensitivity using the GF-AFC substrate for detecting viable cells was determined by mixing serial dilutions of HeLa cells with the substrate in a 96-well plate. The plate was incubated for 30 min before recording fluorescence. The assay consistently demonstrated a sensitivity of between 20 and 50 viable cells per well using signal to noise ratios with a practical limit of sensitivity of 3 as derived from Zhang et al. [15] (Fig. 6).

A similar sensitivity determination was done using the bis-AAF-R110 substrate and serial dilutions of a known number of HeLa cells lysed by sonic disruption. The assay consistently demonstrated a sensitivity of between 10 and 20 compromised cells per well using signal to noise ratios as described previously (Fig. 6).

Utility of combining the substrates in an assay reagent

The ability to detect AFC and R110 fluorescence from both the live and the dead cell markers, respectively, in the same sample was demonstrated using mixtures of viable cells and extract from a known number of disrupted cells. Samples were prepared in a 96-well plate to contain the equivalent of 10,000 Jurkat cells/well composed of mixtures to represent 100–0% viability. Reagent containing

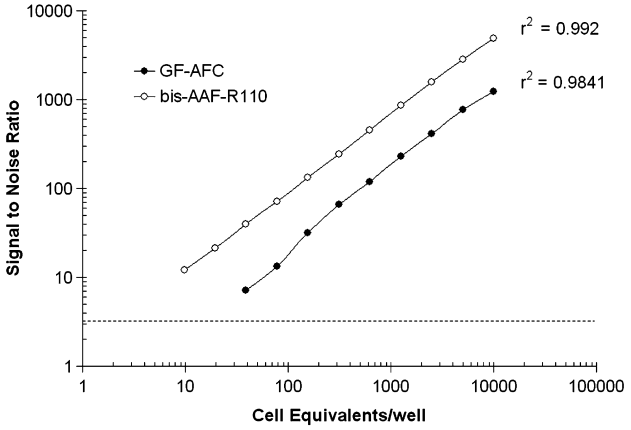

Fig. 6. Cytotoxicity and viability sensitivities in a HeLa cell titration assay after 30 min of reagent contact. Treated or untreated cells were twofold serially diluted in RPMI 1640 + 10% FBS from 10,000 to 10 cells/well. Medium alone served as the background control. The dashed line indicates the practical limit of assay sensitivity for the measures.

GF-AFC and bis-AAF-R110 substrates was added and incubated for 30 min, and fluorescence was recorded at the appropriate wavelengths. The results in Fig. 7 show an inverse relationship between the fluorescence from the viable cell and that from the dead cell markers. There is a linear relationship between the fluorescence (% of maximal response) and the percentage viability for both the viable and the dead cells markers. The sensitivity of detection of viable and dead cells in the multiplex format is approximately 200 cells (2% of 10,000 cells/well).

The multiplex format assay procedure was tested using a diverse selection of cell types shown in Table 3. Fig. 8 shows a representative example experiment using the multiplex protocol to measure viable and dead cells after extended treatment with serial dilutions of staurosporine. Increasing concentrations of staurosporine resulted in a decrease in AFC fluorescence, indicating a decrease in cell viability. In the same wells, an increase in luminescence indicated an increase in the number of dead cells. The

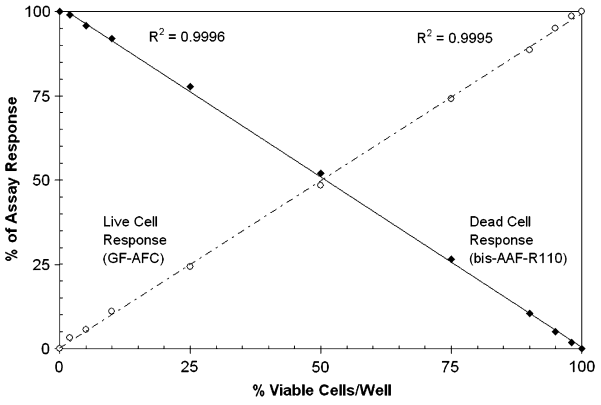

Fig. 7. Multiplexed viability and cytotoxicity assay demonstrates a ratiometric response with practical sensitivities of about 200 viable or dead cells in a total population of 10,000 cells.

Table 3
Cell lines representing the NCI-60 panel used to validate the assay

Cell Line	Sex	Age	Histology	Source/origin
HCT	M	>18	Carcinoma	Colon
HL-60	F	36	Promyleocytic	PBL leukemia
SK-MEL-28	M	51	Melanoma	Melanoma
MCF-7	F	69	Adenocarcinoma	Mammary
PA-1	F	12	Teratocarcinoma	Ovary
ACHN	M	22	Carcinoma	Kidney
PC-3	M	62	Adenocarcinoma	Prostate
DU-145	M	69	Carcinoma	Prostate
NCI-H226	M	na	Squamous	Lung
LN-18	M	65	Glioblastoma	Brain
HeLa	F	na	Carcinoma	Cervix
Jurkat	M	na	T-cell leukemia	Lymphocyte
Hek293	na	<1	Transformed	Kidney
HepG2	M	15	Hepatocarcinoma	Liver
NK-92CI	M	50	Lymphoma	NK cell
U937	M	37	Histocystic lymphoma	Monocyte

EC_{50} values determined from the viable cell marker fluorescence and the dead cell marker luminescence were similar.

The dead cell marker protease activity can be measured using either the luminogenic AAF-aminoluciferin or the fluorogenic bis-AAF-R110 substrates. A multiplex experiment combining the GF-AFC substrate for the viable cell marker and the bis-AAF-R110 substrate for the dead cell marker is illustrated in Fig. 9. Jurkat cells at a density of 10,000 cells/well were treated with various concentrations of ionomycin to induce cell death. The results show a strong EC_{50} correlation between the increase in the R110 signal (indicating more dead cells) and a decrease in the AFC signal (indicating fewer viable cells) with increasing dosage of the cytotoxic agent.

Additional multiplexed content

The multiplex protocol using the GF-AFC substrate and the bis-AAF-R110 substrate can be combined with other spectrally distinct fluorescent assays or with luminescent assays. Fig. 10 shows the results of combining the measurement of caspase-3 activity using a luminescent assay with the fluorescent assays to measure the viable and dead markers. Jurkat cells at a density of 10,000 cells/well were induced to undergo apoptosis by serial dilutions of anti-Fas mAb for 5 h. GF-AFC and bis-AAF-R110 substrates were delivered into the wells in a 1/10th volume and viability and cytotoxicity was determined by measuring their respective signals. Caspase-Glo® 3/7 was delivered into the wells in an equal volume and luminescence from caspase activation was measured. The apoptotic response was reflected by an increase in caspase-3/7 activity whereas the cytotoxic responses were marked by dose-dependent decreases in AFC fluorescence and increases in R110 fluorescence compared to vehicle controls. Because caspase activation precedes changes in membrane integrity, the apparent potency of the

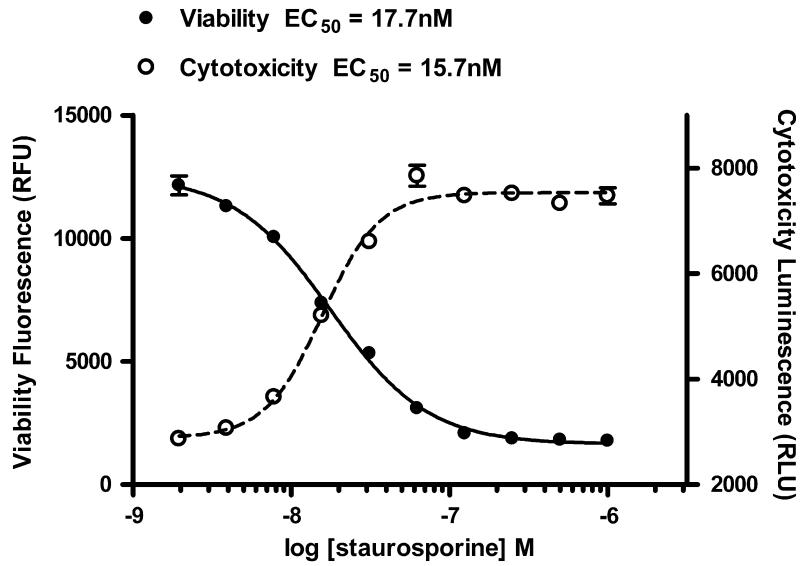

Fig. 8. Fluorescent and luminescent multiplexed viability and cytotoxicity assay. Staurosporine was two fold serially diluted and added to Jurkat cells for a 48-h exposure period. GF-AFC and Ala-Ala-Phe aminoluciferin reagents were prepared and added and signals were measured in a sequential manner. Replicate data for AFC fluorescence and luminescence were fitted using GraphPad Prism software™.

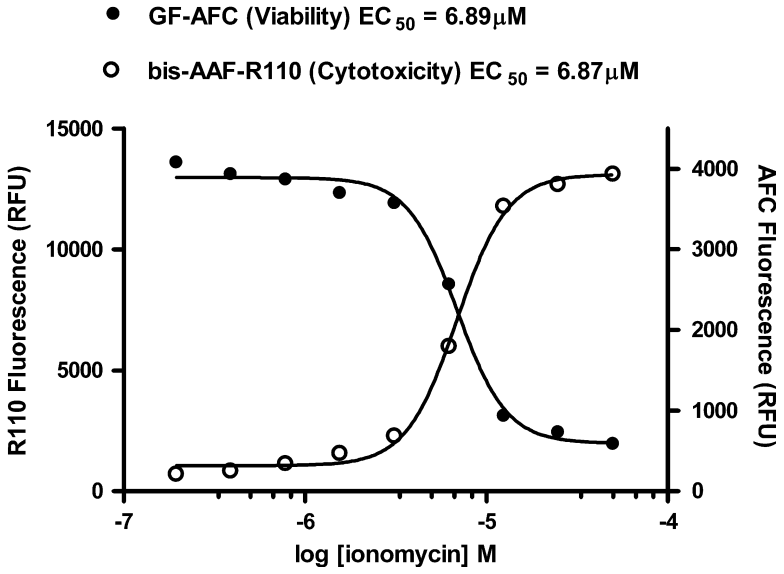

Fig. 9. Double fluorescence multiplexed viability and cytotoxicity assay. Jurkat cells were treated with serial dosages of ionomycin for 6 h. A multiplexed reagent was simultaneously added to cells and fluorescent signals were recorded with a BMG PolarStar™.

apoptosis-inducing compounds was greater for caspase than the observed correlates of cell death.

Discussion

Both drug discovery and primary research efforts continue to utilize increasingly sophisticated cell model systems. The obligate need to measure cell number and viability in these in vitro systems after experimental manipulation is well appreciated. This requirement is necessary to verify the validity of measures and normalize these responses within the context of complex biological systems.

A number of new, nondestructive chemistries have been developed to address cytotoxity in cell culture populations [16–19]. The fluorescent dye reduction chemistries are sensitive and useful but are incompatible in homogeneous multiplexes with other convenient nondestructive viability measures due to shared resazurin substrate useage and spectral overlap. Furthermore, the unreduced resazurin dye used in the assays may quench or skew downstream multiplex data sets if not uniformly reduced with thiols prior to second assay data collection [20].

The commercially available, nondestructive, luminescent cytotoxicity chemistries provide for rapid detection

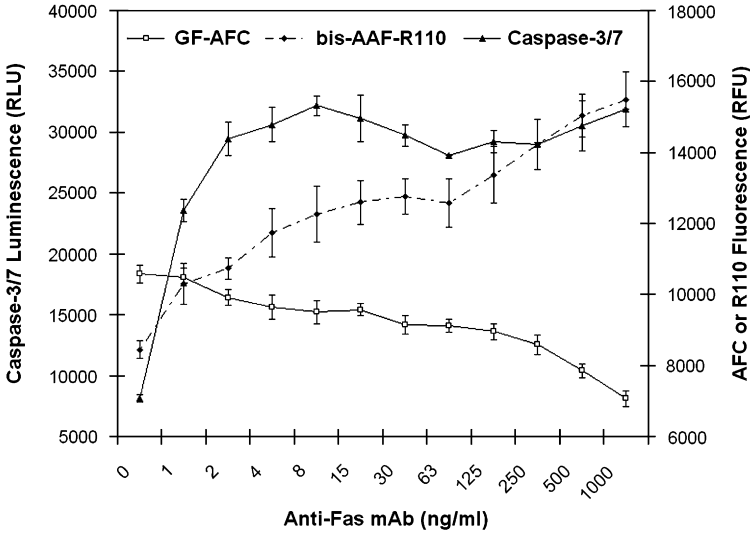

Fig. 10. Multiplexed cell viability, cytotoxicity, and caspase-3/7. Anti-Fas mAb treatment plotted as raw fluorescence or luminescence.

of leaked cytoplasmic enzymes; however, they not only have limited cytotoxicity marker stability but also demonstrate poor luciferase signal half-lives. Marker instability can lead to underestimation of cytotoxicity while short assay signal stability can contribute significant plate to plate variation with batch addition of reagent in multiplate screens. In addition, these assays cannot be homogeneously multiplexed with downstream luminescent applications for reporter gene modulation, caspase activation, or other activity assays.

We have developed homogeneous methods to simultaneously detect viable and dead cells in the same sample by selective measurements of different protease activities using spectrally distinct fluorogenic peptide substrates or combinations of fluorogenic peptides with luminogenic peptides. Although the precise identity of the protease(s) responsible for these activities is unknown, they appear to be abundant, constitutive, consistently measurable, and conserved in mammalian cell lines. Regardless of detection method used, the measures represent inverse correlates of viability and cytotoxicity which can be used as counter measures of the observed phenotype. This complimentarity allows for ratiometric determinations of viability and cytotoxicity to normalize the assay response for cell number. Divergence from the ratiometric response can occur with proliferation in the absence of cytotoxicity or by fluorescence or luminescence interference with one of the signals. Also, because of enzymatic half-life constraints, cytotoxicity (and inverse complementarity) might be impacted if assay measurements are taken at time points several hours after the cytotoxic event. Nevertheless, the reagents were designed to provide sensitive homogeneous methods suitable for high-throughput screening applications. When coupled with compatible downstream multiplexed assays, this technology allows for higher informational content on a per-well basis.

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