Monitor the Ratio of Live and Dead Cells Within a Population: MultiTox-Fluor Multiplex Cytotoxicity Assay

ABSTRACT | Here we report the development of a novel, homogeneous, single-addition reagent that allows researchers to measure the relative number of live and dead cells in culture wells. This assay technology gives ratiometric, inversely proportional values of viability and cytotoxicity that are useful for normalizing data to cell number. Lastly, this reagent is fully compatible with additional fluorescent and luminescent chemistries.

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INTRODUCTION

Cell-based assays are an important tool for contemporary biological research and drug discovery because of their predictive potential for in vivo applications. However, the same cellular complexity that allows the study of regulatory elements, signaling cascades, or test compound biokinetic profiles, can also complicate data interpretation by inherent biological variation. Therefore, researchers often need both to normalize and confirm the validity of assay responses by using the viability of cells in culture after experimental manipulation.

A multitude of assay chemistries are currently employed to determine viability or cytotoxicity in cell-based assays (1). Traditionally, these assay chemistries have been used in parallel assay plates separate from the primary response assay. This approach is typically not cost-effective, as it duplicates the research effort and consumable expense while verifying only the primary response. Previously, we described various multiplexed applications for merging conventional viability or cytotoxicity measurements with the primary response chemistry in the same assay well (2). These methods allow response confirmation and intrawell normalization. Although useful, these applications demonstrate technical drawbacks associated with the absorbance profile of resazurin, resulting in varying degrees of color quenching of fluorescence or luminescence.

Here we report the development of a novel, homogeneous, single-addition reagent that allows researchers to measure the relative number of live and dead cells in culture wells. This assay technology gives ratiometric, inversely proportional values of viability and cytotoxicity that are useful for normalizing data to cell number. Lastly, this reagent is fully compatible with additional fluorescent and luminescent chemistries.

ASSAY DESIGN AND CHEMISTRY

The MultiTox-Fluor Multiplex Cytotoxicity Assay[a] (Cat.# G9200) is analogous to other assays that use membrane integrity changes to measure cell viability or cytotoxicity. However, instead of dye exclusion or lactate dehydrogenase (LDH) release, the MultiTox-Fluor Assay technology simultaneously measures two distinct protease activities as markers for cell viability or cytotoxicity. Procedurally, two fluorogenic substrates are delivered to cell culture wells in a physiological buffer (Figures 1 and 2).

The live-cell protease activity is restricted to intact viable cells and is measured using the fluorogenic, cell-permeant, peptide substrate, Gly-Phe-7-amino-4 trifluoromethyl coumarin (GF-AFC). The substrate enters intact cells where it is cleaved to generate a fluorescent signal proportional to the number of living cells. This live-cell protease activity marker becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium. The second substrate, bis-(Ala-Ala-Phe)-rhodamine 110 (bis-AAF-R110), is a cell-impermeant, fluorogenic peptide substrate that measures dead-cell protease activity that has been released from cells that have lost membrane integrity.

MULTIPLEX SIGNAL SEPARATION AND MEASUREMENT

The MultiTox-Fluor Multiplex Cytotoxicity Assay uses distinct fluorescent products that can be optically separated. Like many fluorogenic dyes, there is modest overlap in the absorption and emission profiles of AFC and R110 beyond their optimal peaks. This overlap is overcome by choosing bandpass filter sets on fluorometers that separate the respective signals while remaining near the excitation and emission signal peaks. The liberated AFC fluorophore can be measured using a fluorometer at an excitation of 400nm and emission of 505nm (3). The R110 product can be measured at an excitation of 485nm and emission of 520nm (4; Figure 3). It is important to note that deviation from the suggested wavelengths may adversely affect assay sensitivity and performance.

The MultiTox-Fluor Assay simultaneously measures two distinct protease activities as markers for cell viability and cytotoxicity.

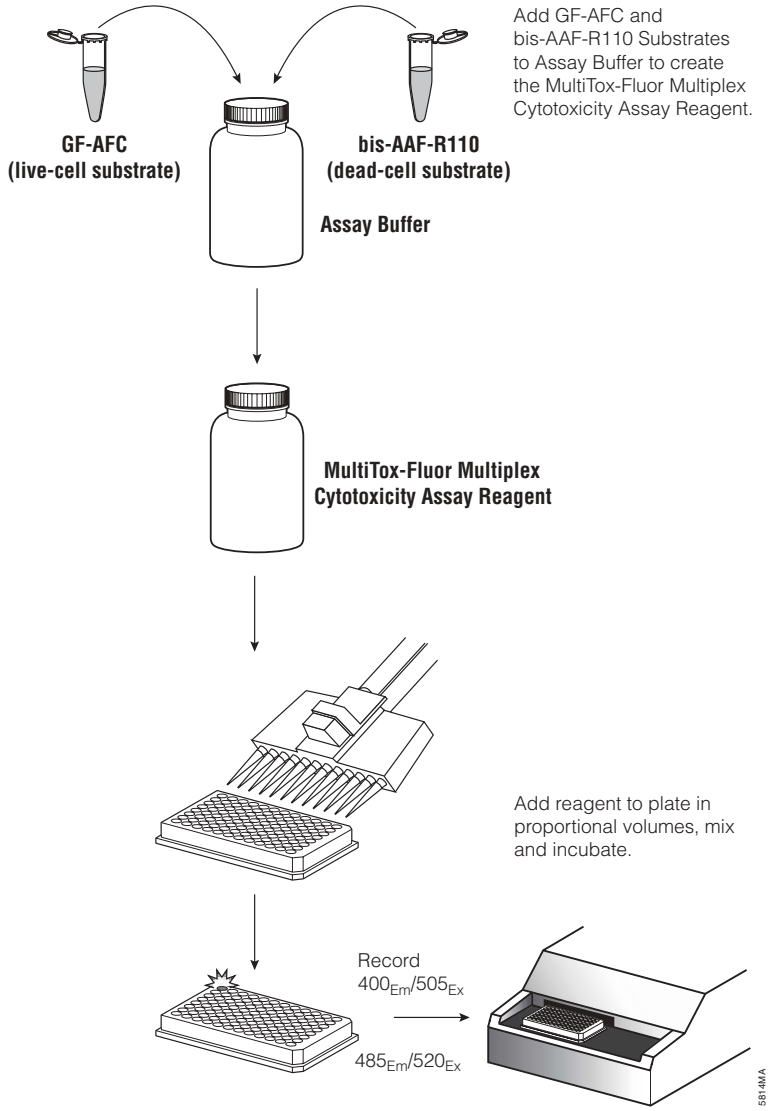

Figure 1. Schematic diagram of the MultiTox-Fluor Multiplex Cytotoxicity Assay. The assay reagent is created by adding the fluorogenic peptide substrates [GF-AFC (live-cell protease substrate) and bis-AAF-R110 (dead-cell protease substrate)] to the assay buffer. This reagent can then be added to a multiwell plate. After at least 30 minutes of incubation at 37°C, the resulting fluorescent signals may be measured at an excitation of 400nm and an emission of 505nm (live-cell signal), then at an excitation of 485nm and an emission of 520nm (dead-cell signal).

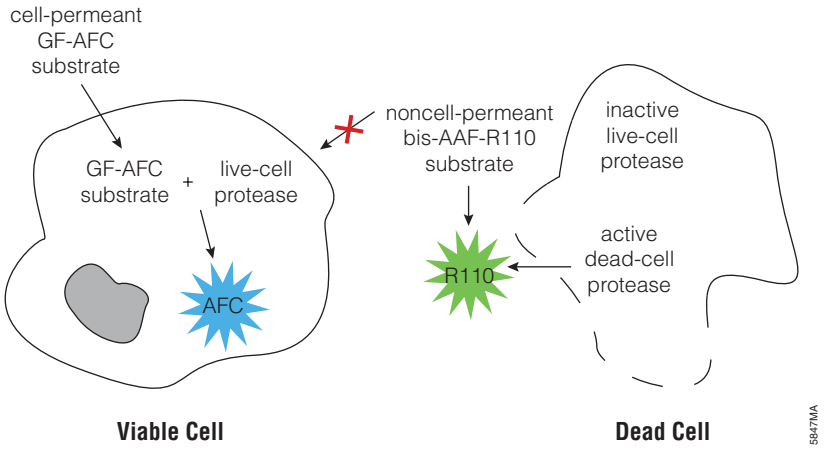

Figure 2. The biology of the MultiTox-Fluor Multiplex Cytotoxicity Assay. The GF-AFC Substrate can enter live cells where it is cleaved by the live-cell protease to release AFC. The bis-AAF-R110 Substrate cannot enter live cells but instead can be cleaved by the dead-cell protease activity to release R110.

These measures of proteolytic activity mirror the data obtained from enzyme release, resazurin reduction, ATP quantitation or dye exclusion assays (Figure 4).

THE RATIOMETRIC RESPONSE

The greatest advantage derived from measuring the activity from both live and dead cells by the MultiTox-Fluor Multiplex Cytotoxicity Assay is that the measures are inversely proportional and complementary (Figure 4). Simply stated, when one fluorescence marker response is high, the other will be low compared to viability or cytotoxicity controls. This provides a ratiometric response that can be used to normalize the data, irrespective of cell number. Ultimately, these independent measures serve as assay controls and can help identify and correct errors caused by pipetting, differential growth patterns or interference with assay chemistry.

SENSITIVITY

The MultiTox-Fluor Multiplex Cytotoxicity Assay directly measures two different protease activities with rapid catalytic cleavage rates. This allows rapid accumulation of fluorescent product in the cell culture well. After a 30-minute incubation, even modest declines in viability (5%) can be measured in most cell types when using 10,000 cells/well (Figure 4). Increasing incubation times up to three hours often increases the signal window and detects cytotoxicity of as little as 2% (200 cells) of the population. Because the assay reagent does not affect viability, multiple time point measurements can be taken until the highest level of sensitivity is obtained.

CORRELATION WITH CONVENTIONAL VIABILITY AND CYTOTOXICITY ASSAYS

The MultiTox-Fluor Multiplex Cytotoxicity Assay represents a novel method for measuring viability and cytotoxicity of cells in culture. However, because the assay is designed to measure distinct changes in membrane integrity, data derived from the assay correlate well with existing methodologies. Therefore, these measures of proteolytic activity mirror the data obtained from enzyme release, resazurin reduction, ATP quantitation or dye exclusion assays (Figure 5).

ADDITIONAL DOWNSTREAM MULTIPLEXES

The MultiTox-Fluor Multiplex Cytotoxicity Assay Reagent can be delivered to wells in various volumes by adjusting the substrate concentration in the reagent. For instance, a 2X reagent can be prepared and added at a volume

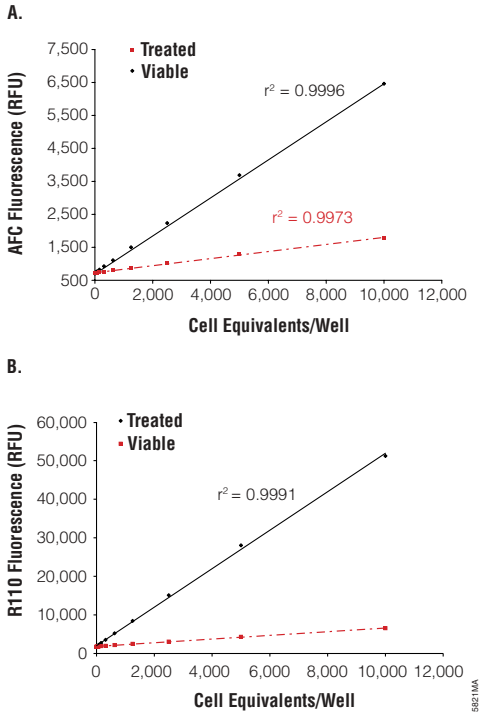

Figure 3. The MultiTox-Fluor Multiplex Cytotoxicity Assay measures two distinct proteolytic activities that are mutually restricted to either viable or compromised cells. A pool of Jurkat cells was divided into two fractions. One fraction was subjected to treatment to simulate cytotoxicity, whereas the other was left untreated. Both fractions were twofold serially diluted in RPMI 1640 + 10% fetal bovine serum. Medium with serum served as the no-cell control. Single-substrate reagents were created by adding either GF-AFC or bis-AAF-R110 to buffer. Each was added to cells and incubated for 30 minutes at 37°C prior to measuring fluorescence using a BMG POLARstar reader. **Panel A.** Differential fluorescence profile of the live-cell substrate (GF-AFC). **Panel B.** Differential fluorescence profile of the dead-cell substrate (bis-AAF-R110).

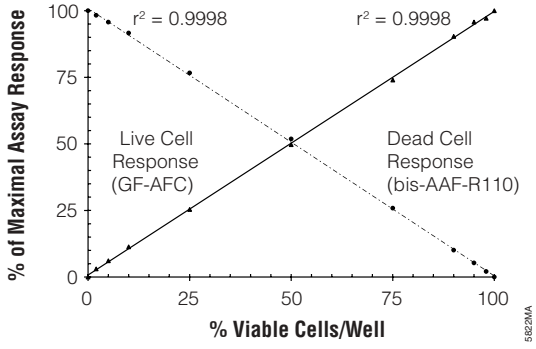

Figure 4. The ratiometric response obtained from the MultiTox-Fluor Assay. A pool of Jurkat cells was adjusted to 100,000 cells/ml, then divided into two fractions. One fraction was compromised by treatment to induce cytotoxicity; the other untreated. The two fractions were then combined in various proportions to simulate varying viabilities from 100% to 0%. Ten thousand cell equivalents were added to each well in 100µl volumes, followed by an equal addition of the MultiTox-Fluor Assay Reagent. After a brief orbital shaking to ensure dispersion of the Reagent, the plate was incubated at 37°C for 30 minutes prior to measuring fluorescence using the BMG POLARstar plate reader. The data were normalized as a percentage of the maximal response. The dashed line represents fluorescence generated in the presence of dead cells, and the unbroken line represents fluorescence in the presence of live cells.

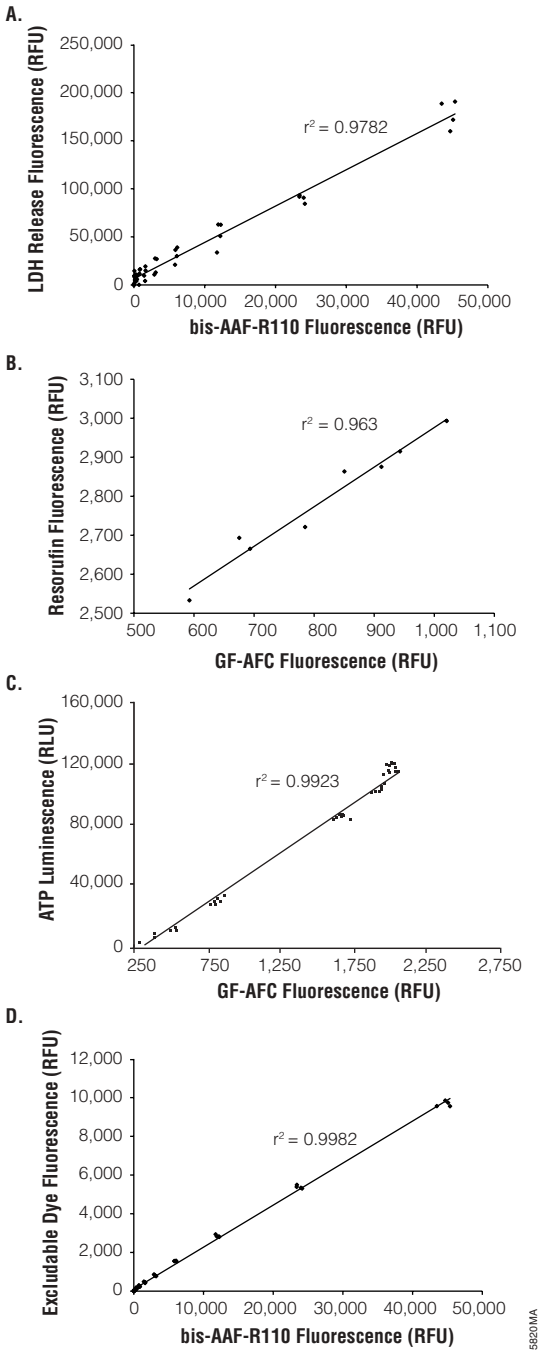

Figure 5. The MultiTox-Fluor Assay data correlate with other conventional measures of viability and cytotoxicity. U937, Jurkat, and HL-60 cells were subjected to various treatments that induced different degrees of cytotoxicity as described in the *MultiTox-Fluor Multiplex Cytotoxicity Assay Technical Bulletin* #TB348. Parallel plates were prepared and cytotoxicity and viability determined by MultiTox-Fluor Assay Technology, CytoTox-ONE™ Assay (**Panel A**), CellTiter-Blue® Assay (**Panel B**), CellTiter-Glo® Assay (**Panel C**) or by ethidium homodimer incorporation (**Panel D**, Molecular Probes). Resulting fluorescence signals were plotted against MultiTox-Fluor Assay technology readings.

equal to the cells, or a more concentrated reagent can be prepared and added at a volume 1/10 that of the culture medium. This smaller volume addition provides flexibility and accommodates the addition of other reagents that may be required for a second multiplex assay such as reporter activity or caspase activation assays. Because the substrates are essentially colorless and inert with respect to influencing viability, a large number of multiplex options exist using either spectrally distinct fluorophores or luminescent assays (Figure 6).

CELL-SPECIFIC ASSAY RESPONSES

There is significant diversity with regard to morphology and lineage among cell lines used in routine biological research. Different cell lines have different reductive capacities or contain different levels of enzyme markers. These factors may influence assay incubation times or experimental sensitivity with conventional viability or cytotoxicity chemistries. The MultiTox-Fluor Multiplex Cytotoxicity Assay was validated using selected cell lines representative of the National Cancer Institute-60 panel (5) and found to work well in various necrosis or apoptosis models (Table 1).

A large number of multiplex options exist using either spectrally distinct fluorophores or luminescent assays (Figure 6).

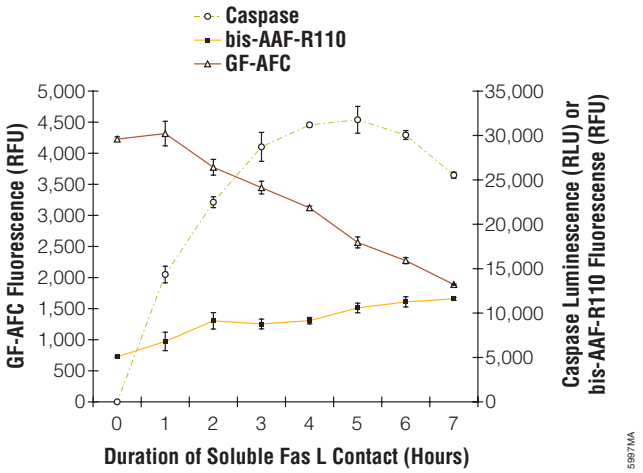

Figure 6. The MultiTox-Fluor Assay can be multiplexed with other assays. Jurkat cells were plated at a density of 10,000 cells per well in 50µl volumes in RPMI 1640 + 10% fetal bovine serum (FBS) and allowed to equilibrate for 2 hours at 37°C in 5% CO_2. Soluble rhFas L (R&D Systems) was diluted to 400ng/ml in RPMI 1640 + 10% FBS and added in 50µl volumes to replicate assay wells every hour for a period of 7 hours. MultiTox-Fluor Reagent was prepared as a 20X reagent and delivered in a 10µl volume. The plate was mixed by orbital shaking, then returned to the incubator for 30 minutes before reading the fluorescent signals on a BMG POLARstar plate reader. Caspase-Glo® 3/7 Reagent was then added in an additional 100µl volume and resulting luminescence measured after a 10-minute incubation using the BMG POLARstar plate reader. The resulting signals from the cytotoxicity, viability and background-subtracted caspase-3/7 responses were then plotted as a function of time.

CONCLUSIONS

The MultiTox-Fluor Multiplex Cytotoxicity Assay uses a simple, sensitive and scalable chemistry to determine the degree of viability and cytotoxicity within cell culture wells after experimental treatment. The live-cell and dead-cell proteolytic measures in the MultiTox-Fluor Multiplex Cytotoxicity Assay correlate well with conventional measures of cell viability and act as internal controls while providing a ratiometric response, which is useful for normalization purposes. Furthermore, the assay system provides the additional functionality and flexibility to enable multiplexing with other assays.

Table 1. The MultiTox-Fluor Assay was validated using cell lines representative of the diversity in the National Cancer Institute-60 (NCI-60) panel.

Cell Line	Sex	Age	Histology	Source/Origin
HCT-116	M	>18	carcinoma	colon
HL-60 (TB)	F	36	promyleocytic	PBL leukemia
SK-MEL-28	M	51	melanoma	melanoma/skin
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	31	carcinoma	cervix
Jurkat	M	na	T-cell leukemia	lymphocyte
HEK 293	N/A	fetal	transformed	kidney
HepG2	M	15	hepatocarcinoma	liver
NK-92CI	M	50	lymphoma	NK cell
U937	M	37	histocystic lymphoma	monocyte

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PROTOCOL

- *MultiTox-Fluor Multiplex Cytotoxicity Assay Technical Bulletin* #TB348, Promega Corporation.
  (www.promega.com/tbs/tb348/tb348.html)

ORDERING INFORMATION

Product	size	Cat.#
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
	5 × 10ml	G9201
	2 × 50ml	G9202

For Laboratory Use.

[a]Patent Pending.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.