

# Evaluation of Impermeant, DNA-Binding Dye Fluorescence as a Real-Time Readout of Eukaryotic Cell Toxicity in a High Throughput Screening Format

Lucius Chiaraviglio and James E. Kirby

Department of Pathology, Beth Israel Deaconess Medical Center, Boston, Massachusetts.

## ABSTRACT

Interpretation of high throughput screening (HTS) data in cell-based assays may be confounded by cytotoxic properties of screening compounds. Therefore, assessing cell toxicity in real time during the HTS process itself would be highly advantageous. Here, we investigate the potential of putatively impermeant, fluorescent, DNA-binding dyes to give cell toxicity readout during HTS. Amongst 19 DNA-binding dyes examined, three classes were identified that were (1) permeant, (2) cytotoxic, or (3) neither permeant nor cytotoxic during 3-day incubation with a macrophage cell line. In the last class, four dyes (SYTOX Green, CellTox Green, GelGreen, and EvaGreen) gave highly robust cytotoxicity data in 384-well screening plates. As proof of principle, successful combination with a luminescence-based assay in HTS format was demonstrated. Here, both intracellular growth of *Legionella pneumophila* (luminescence) and host cell viability (SYTOX Green exclusion) were assayed in the same screening well. Incorporation of membrane-impermeant, DNA-binding, fluorescent dyes in HTS assays should prove useful by allowing evaluation of cytotoxicity in real time, eliminating reagent addition steps and effort associated with endpoint cell viability analysis, and reducing the need for follow-up cytotoxicity screening.

## INTRODUCTION

Traditionally, eukaryotic cell toxicity is measured in an “endpoint” assay. Specifically, a reagent is added after a defined time period to measure a metabolic or physical property. Classic examples include measurement of lactate dehydrogenase release through addition of substrate, cellular ATP through use of ATP-dependent luminescent chemistry, and permeability to membrane-impermeant dyes such as propidium iodide.<sup>1</sup> These assays have proven extremely useful. However, their amenability to a high throughput format is suboptimal. Specifically, as endpoint assays, they require several additional steps to be performed at the end of the screening process. Many cytotoxicity assays also have a time-constrained readout; for example, measurement of an

enzyme-dependent color reaction that varies with time. Therefore, multiplied over the large number of screening plates in high throughput screening (HTS), the additional effort required for these endpoint assays may prove considerable or impractical.<sup>2–4</sup>

Accordingly, the ability to add cytotoxicity test reagents during assay setup, akin to inclusion of fluorescent probes in real-time polymerase chain reaction (PCR) assays,<sup>5–7</sup> would confer great advantage. Impermeant DNA-binding dyes from a theoretical standpoint would seem ideal for this purpose. These dyes enter and bind to nuclear DNA as dying eukaryotic cells lose membrane permeability. Several demonstrate little to no solution fluorescence and greatly increased quantum yield when bound to DNA.<sup>8,9</sup> Therefore, fluorescent signal correlates with loss of eukaryotic cell viability and can be normalized to positive (*i.e.*, permeabilized) and negative controls to give a fractional measure of cell death or cell viability.

The use of DNA-binding dyes in this way will depend on satisfying several conditions: first, that the dyes remain nonpermeable for an extended period of time; second, that the dyes themselves are non-toxic; and third, that dyes have spectral characteristics that allow efficient detection in available high throughput microplate readers. We therefore sought to examine a large number of commercially available, impermeant, DNA-binding dyes to assess suitability for use as real-time HTS cytotoxicity probes.

## MATERIALS AND METHODS

### Reagents

SYTOX Blue™, SYTOX Green™, SYTOX Orange™, SYTOX Red™, TOTO-3™, BOBO-3™, POPO-1™, YO-PRO-1™, YO-PRO-3™, SYTO-9™, SYTOX AADvanced™, YOYO-1™, YOYO-3™, and propidium iodide were purchased from Life Technologies (Grand Island, NY). CellTox Green™ was purchased from Promega (Madison, WI); DRAQ7™ was purchased from Biostatus Limited (Leicestershire, United Kingdom). RedDot2™, EvaGreen™, GelGreen™, and GelRed™ were purchased from Biotium, Inc. (Hayward, CA). DRAQ7, EvaGreen, GelGreen, and GelRed were purchased as aqueous stock solutions. The remaining dyes were purchased as DMSO stock solutions. Dyes were diluted in experiments to final concentrations listed in *Table 1*. Saponin, levofloxacin, azithromycin, and DMSO (cell culture grade) were purchased from Sigma-Aldrich (St. Louis, MO).

**Table 1. Summary of Fluorescent Dyes Tested by Microscopy and/or Plate Reading**

Dye	Final conc. <sup>a</sup>	Permeability/ cytotoxicity characteristics <sup>b</sup>	Excitation optimum (range)	Emission optimum (range)	Dichroic mirror cutoff or beam splitter <sup>c</sup>	Excitation filter, nm/bandwidth <sup>c</sup>	Emission filter, nm/bandwidth <sup>c</sup>
POPO-1	0.1 μM	Nonpermeable, nontoxic	434 (395–451)	456 (440–513)	Beam splitter (BS550, barcode 401)	420/8 (Photometric 420, barcode 308)	475/8 (Photometric 475, barcode 309)
SYTOX Blue	1 μM	Nonpermeable, nontoxic	444 (405–462)	480 (452–521)	Beam splitter (BS550, barcode 401)	420/8 (Photometric 420, barcode 308)	475/8 (Photometric 475, barcode 309)
YOYO-1	0.1 μM	Cytoplasmic permeable, non-toxic	491 (441–507)	509 (491–573)	ND	ND	ND
YOPRO-1	0.1 μM	Cytoplasmic permeable, nontoxic	491 (442–507)	509 (491–570)	ND	ND	ND
GelGreen	1×	Nonpermeable, nontoxic	270 (200–300) 510 (460–530) <sup>d</sup>	530 (510–565)	505 (FITC, barcode 403)	485/14 (FITC 485, barcode 102)	535/25 (FITC 535, barcode 206)
EvaGreen	0.5×	Nonpermeable, nontoxic	500 (455–520)	530 (505–565)	505 (FITC, barcode 403)	485/14 (FITC 485, barcode 102)	535/25 (FITC 535, barcode 206)
SYTOX Green	125 nM	Nonpermeable, nontoxic	504 (460–522)	523 (505–588)	505 (FITC, barcode 403)	485/14 (FITC 485, barcode 102)	535/25 (FITC 535, barcode 206)
CellTox Green	0.5×	Nonpermeable, nontoxic	512 (460–522)	532 (524–590)	505 (FITC, barcode 403)	485/14 (FITC 485, barcode 102)	535/25 (FITC 535, barcode 206)
Propidium iodide	30 μM	Toxic and permeable after 1 day of incubation	536 (470–581)	617 (579–652)	555 (BODIPY TMR, barcode 405)	530/10 (YFP 530, barcode 221)	650/40 (Custom 650, barcode 227)
SYTOX AADvanced	1 μM	ND	546 (456–603)	647 (596–732)	555 (BODIPY TMR, barcode 405)	530/10 (YFP 530, barcode 221)	650/40 (Custom 650, barcode 227)
SYTOX Orange	0.5 μM* 0.25 μM <sup>†</sup>	Nonpermeable, nontoxic	547 (505–603)	570 (545–614)	555 (BODIPY TMR, barcode 405)	535/25 (FITC 535, barcode 206)	570/8 (Photometric 570, barcode 318) or 590/20 (Rhodamine 590, barcode 217)
BOBO-3	0.1 μM	Cytoplasmic permeable, nontoxic	570 (516–602)	602 (579–652)	ND	ND	ND
YOPRO-3	0.1 μM	Cytoplasmic permeable, nontoxic	612 (547–637)	631 (609–695)	Beam splitter (BS550, barcode 401)	600/8 (Photometric 600, barcode 319)	650/50 (no label, barcode 510)
YOYO-3	0.1 μM	Cytoplasmic permeable, nontoxic	612 (556–633)	631 (606–687)	ND	ND	ND
DRAQ7	3 μM	Nonpermeable, nontoxic	633 (488–680)	678 (650–755)	658 (Cy5, barcode 420)	600/8 (Photometric 600, barcode 319)	665/7.5 (APC, barcode 205)
TOTO-3	0.1 μM	ND	642 (577–667)	660 (637–724)	658 (Cy5, barcode 420)	600/8 (Photometric 600, barcode 319)	665/7.5 (APC, barcode 205)
SYTOX Red	5 nM	ND	640 (584–664)	658 (635–721)	658 (Cy5, barcode 420)	600/8 (Photometric 600, barcode 319)	665/7.5 (APC, barcode 205)
GelRed	1×	Nonpermeable, nontoxic	270 (200–300) 515 (470–560) <sup>e</sup>	595 (555–650)	555 (BODIPY TMR, barcode 405)	520/25 (Custom 520, barcode 506)	590/20 (Rhodamine 590, barcode 217)
RedDot2	1×	Nonpermeable, nontoxic	~ 610 (488–647) <sup>f</sup>	695 (647–760)	658 (Cy5, barcode 420)	600/8 (Photometric 600, barcode 319)	720/8 (Photometric 720, barcode 322)

<sup>a</sup>Concentration used was for both microscopy and plate reading unless otherwise indicated (\*microscopy; <sup>†</sup>plate reading; <sup>‡</sup>plate reading, tested only in white microplate dishes). Where fluorophore concentration was not available (i.e., proprietary), concentration used is indicated relative to the manufacturer's recommended 1× concentration.

<sup>b</sup>Characteristics detected by microscopy.

<sup>c</sup>Perkin-Elmer designations for dichroic mirrors, filters, and beam splitters indicated in parentheses.

<sup>d</sup>Second peak optimal.

<sup>e</sup>First peak has optimal absorbance, but only the second peak could be tested with available filters.

<sup>f</sup>A greater absorption peak exists at ~660 nm, but is not optimal for fluorescent excitation.

ND, not determined.

## Cell Culture

J774A.1 cells (ATCC, Manassas, VA) were passaged in suspension in RPMI 1640 medium (Corning Cellgro, Manassas, VA) containing 9% iron-supplemented calf serum (Atlanta Biologicals, Flowery Branch, GA). For microscopy experiments, J774 cells were plated in the same medium on dishes containing embedded #1.5 thickness, German glass cover slips (catalog # P35G-1.5-20-C; MatTek Corp., Ashland, MA) at approximately  $5 \times 10^7$  cells/cm<sup>2</sup>. For microplate experiments, J774 cells were resuspended in the same medium, lacking phenol red and supplemented with 100 µg/mL thymidine, and plated at approximately  $5 \times 10^7$  cells/cm<sup>2</sup> onto black (Corning 3709) or white (Corning 3570) opaque, sterile, tissue culture-treated, 384-well plates. This plating density equates to  $5 \times 10^4$  J774 cells/well or  $1.92 \times 10^6$  cells per 384-well dish.

## Microscopy

For microscopic assessment of dye permeability and cytotoxicity, coverslips were examined using a Nikon Eclipse Ti™ inverted epifluorescence microscope with standard DAPI, FITC, TRITC, and Cy5 filter cubes. Alterations in J774 cell morphology (cell loss, change in shape) were assessed by phase contrast microscopy. Dye permeability was scored based on fluorescence of the cell nucleus and cytoplasm (within 4 h after dye addition, then on days 1 and 2, and then on day 3 both before and after permeabilization of cells with saponin). Saponin was added to culture wells in one-third volume of additional medium without washing to achieve a final concentration of 0.15%. Microscopic images were captured using a CCD camera (QImaging, Surrey, BC). Paired fluorescent images for each dye (with or without saponin treatment) were processed in parallel in Photoshop CS6™ (Adobe, San Jose, CA) so that contrast adjustments would be applied equally.

## Microplate Dye Evaluation

For *Z'* factor comparison, dyes were added at the final concentrations indicated in Table 1 to J774A.1 cells grown in 384-well microplates in a 50 µL well volume. Positive control wells received 0.15% saponin to permeabilize cell membranes, and negative control wells received the same concentration of DMSO solvent (0.2% final concentration). To allow screening of a large number of dyes contemporaneously, 8 positive control wells and 34 negative DMSO control wells were tested for each dye comparison. A larger number of negative control wells was used in order to detect intrinsic dye permeability with greater confidence (i.e., permeability in the absence of saponin). To detect intrinsic solution fluorescence, 8–12 wells lacking J774A.1 cells were also tested for each dye. Plates were incubated for the indicated amount of time at 37°C with 5% CO<sub>2</sub>, and read on an EnVision™ Multi-Label Reader (Perkin Elmer, Waltham, MA). Filter combinations used for each dye are listed in Table 1. *Z'* were calculated as follows:

$$Z' = 1 - \frac{3 \times (\text{Positive control SD} + \text{Negative control SD})}{|\text{Positive control mean} - \text{Negative control mean}|}$$

## Proof of Principle HTS Infection Experiments

J774 cells were seeded in white, Corning #3570, 384-well microplates to achieve approximately 90% confluence on the day of the experiment. Stock solutions (500×) of saponin, levofloxacin, azithromycin, and DMSO control were introduced respectively at 0.1 µL/well by pin transfer using a customized, Epson robot, each into 96 wells in duplicate microplates (thus 192 wells in total for each test compound). Luminescent *Legionella pneumophila*, strain Lp02:*flaA:lux*, a thymidine auxotroph containing the *Photorhabdus luminescens luxCDABE* operon, was originally constructed in the laboratory of Bill Dietrich<sup>10</sup> and provided by Andrew Olive (Harvard Medical School, Boston, MA). Prior to use in infection experiments, *L. pneumophila* was grown as confluent patches on buffered charcoal yeast extract agar plates supplemented with 0.1% α-ketoglutarate and 100 µg/mL thymidine for 1–2 days. Bacteria were then suspended in tissue culture medium supplemented with 100 µg/mL thymidine and SYTOX Green™ and then added, immediately following pin transfer described above, to J774A.1 cells to achieve a multiplicity of infection of approximately one bacterium per two macrophages. Final concentrations of compounds in microplates after pin transfer and addition of bacteria were 0.15% saponin, 10 µg/mL levofloxacin, or 10 µg/mL azithromycin, and 0.2% DMSO and 125 nM SYTOX Green in all wells. Infected 384-well plates were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere and read on an EnVision™ Multi-Label Reader (Perkin Elmer) at indicated time points. See Table 2 for summary of HTS assay setup.

## Dose–Response

An HP D300 high performance dispensing system (Hewlett-Packard, Palo Alto, CA) was used to inoculate test wells with azithromycin in a twofold dilution series ranging from 16 µg/mL to 4 ng/mL final concentration. Experiments were otherwise performed as described in the preceding section. IC<sub>50</sub> values were calculated in Prism 6 (GraphPad Software, Inc., San Diego, CA) using nonlinear, four parameter regression analysis.

## HTS Pilot

Commercial bioactive libraries were from Biomol (now Enzo Life Sciences, Farmingdale, NY), Microsource (Gaylordsville, CT), Sigma-Aldrich, Biofocus (Saffron Walden, United Kingdom); Enzo Life Sciences, Prestwick (Illkirch, France), and Chembridge (San Diego, CA). Compounds were dissolved in DMSO. Experiments were performed in the same manner as described in Table 2 with the following modification. Pin transfer for screening compounds was performed into 384 well screening plates in duplicate. Final concentrations of compounds were usually at either 4 µg/mL or 20 µM. Library arrays were from the ICCB-Longwood collections of known bioactive compounds.<sup>11</sup> Screening compounds were added to columns 1–22 in plates by pin transfer as described above. Column 23 was used for infected negative controls that were not treated with compound, 16 wells per plate. Column 24 contained eight interleaved positive controls each for luminescence (levofloxacin) and fluorescence (saponin). In contrast to proof of principle experiments, control compounds were added

Table 2. High Throughput Assay Protocol Table

Step	Procedure	Value	Description
1	Plate J774A.1 cells	30 $\mu$ L per well	$5 \times 10^4$ J774A.1 cells/well
2	Passage bacteria	<i>Legionella pneumophila</i> strain Lp02-Lux-flaA	Bacterial strain expresses luminescence operon that permits relative quantification of bacterial number over time
3	Incubate J774A.1 cells and bacteria separately overnight	Approximately 20 h	Incubate J774A.1 cells at 37°C with 5% CO <sub>2</sub> to achieve approximately 90% confluence; incubate bacteria at 37°C in ambient air
4	Pin transfer of test compounds	500 $\times$ concentrates in DMSO; 0.1 $\mu$ L/well	Saponin, mammalian cell lytic positive control; levofloxacin and azithromycin bacterial inhibition controls
5	Infection and SYTOX Green addition	20 $\mu$ L per well	Final concentration of $5 \times 10^4$ bacteria/well and 125 nM SYTOX Green
6	Pre-incubation readout of bacterial number	Luminescence	Quantify bacteria by luminescence
7	Pre-incubation readout of mammalian cell lysis	SYTOX Green Fluorescence	Cells that have lost membrane integrity exhibit fluorescent DNA staining
8	Repeat steps 6 and 7 after 1, 2, and 3 day incubation	As in steps 6 and 7	Incubate at 37°C with 5% CO <sub>2</sub> between readings

**Step Notes**

1. Cells suspended in RPMI 1640 medium containing 9% iron-supplemented calf serum without phenol red, supplemented with 100 mg/mL thymidine; plated in Corning #3570 (white) or #3709 (black) dishes (384 wells each), growth area 0.072 cm<sup>2</sup>/well.
2. Transfer a visible amount of bacterial mass from a first passage plate to a second passage plate to create a growth patch (buffered charcoal yeast extract agar supplemented with 100 mg/mL thymidine).
3. Bacteria should develop into lawn prior to harvesting.
4. Control compound 500 $\times$  stock solutions dissolved in DMSO (saponin, 75 mg/mL; levofloxacin, 5.0 mg/mL; azithromycin, 5.0 mg/mL). Pin transfer tip array washed in 20% methanol solution by sonication between uses.
5. Bacteria and SYTOX Green suspended in tissue culture medium and transferred to microplates using a Matrix Technologies/Thermo Fisher Scientific (Cambridge, MA) WellMate programmable multichannel peristaltic pump.
6. Perkin-Elmer EnVision "USLum" protocol used for high-sensitivity luminescence detection.
7. Perkin-Elmer EnVision fluorescence reading with Excitation 485/14, Emission 535/25, Dichroic Mirror 505 nm, minimum gain, minimum transmittance, and "High Concentration Mode" selected to prevent detector saturation.

manually with a multichannel pipette. Modified  $z$  scores were calculated according to the formulae  $z = (x - \mu) / \sigma$  (fluorescence) or  $z = (\mu - x) / \sigma$  (luminescence), where  $x$  is the luminescent or fluorescence readout for each test well, and  $\mu$  and  $\sigma$  are the mean and standard deviation values for the negative control wells within each respective screening plate. We specifically opted not to use the  $\mu$  and  $\sigma$  values from the test wells for comparison, as the expected large number of positive hits in the known bioactive libraries would exaggerate experimental variability and lead to erroneous reduction in  $z$  score values. This strategy has been suggested previously to compensate for a high hit frequency in screening plates.<sup>12,13</sup> The  $\mu$  and  $x$  were reversed in the luminescence  $z$  score formula so that reduction in luminescence would yield a positive value. Scatter plots were created and analyzed using the Vortex software package (Dotmatics, San Diego, CA).

**RESULTS****Permeability and Toxicity**

Fluorescent nucleic acid-binding dyes, described by their manufacturers as cell impermeant, were pre-screened by microscopy for

permeability and toxicity during a 3-day incubation with J774 cells, a murine monocyte/macrophage cell line. In these and subsequent experiments, the positive permeability control was treatment with saponin, a cholesterol-binding detergent, that efficiently permeabilizes eukaryotic cells.<sup>14</sup> Dyes were subsequently categorized into three classes: cytoplasmic-permeable, noncytotoxic (class I); nucleus-permeable, cytotoxic (class II); and nonpermeable, nontoxic (class III) as summarized in Table 1. Representative images for each class of dye are shown in Figure 1.

In the first class, we noted several that stained the cytoplasm of J774 cells during prolonged incubation. This staining occurred without overt morphological alterations, suggesting lack of significant cytotoxicity. Although these dyes appeared to be largely excluded from the cell nucleus, the intensity of cytoplasmic staining appeared greater than or equal to nuclear staining following saponin permeabilization (e.g., Fig. 1, YOYO-3), predicting lack of utility in microplate assays. In contrast, propidium iodide (class II, Fig. 1), a dye used to assess cell death in both eukaryotic and prokaryotic endpoint assays,<sup>15,16</sup> was initially impermeant to J774 cells at up to 1

day of incubation, but then became permeant to J774 cells by 2 days of incubation—that is, staining equal to saponin-treated controls, staining nuclei intensely (Fig. 1 and data not shown). It also caused J774 cells to round up and detach from dishes (Fig. 1), indicating significant cytotoxicity. Importantly, most of the dyes tested proved to be neither overtly permeable nor cytotoxic to J774 cells, and were accordingly candidates for use in real-time cytotoxicity assays.

### Suitability for High Throughput Readout

Therefore, nontoxic, long-term impermeant dyes were tested for their ability to yield robust cytotoxicity data in 384-well black and white opaque tissue culture plates (Table 3). Several dyes with green or orange emission spectra yielded high  $Z' > 0.5$  (SYTOX Green and Orange, CellTox Green, EvaGreen, and GelGreen), generally with somewhat better performance in white dishes. However, dyes with red or far-red emission spectra uniformly performed poorly on a multimode plate reader, despite giving distinct permeability readout by microscopy. Specifically, SYTOX AADvanced, SYTOX Red, and RedDot2 (Table 3) yielded very low signal and therefore little separation

between positive and negative controls. YOPRO-3 and TOTO-3 demonstrated modest but still insufficient separation ( $Z' < 0$ ). Signal-to-noise ratio aside, anomalous readings for DRAQ7 and, to a lesser extent, RedDot2 (higher signal in the absence of J774 cells than in their presence) also raised further concern about reliability. Finally, GelRed saturated the detector under all conditions tested. For blue fluorescent dyes, SYTOX Blue and POPO-1, band-pass filters alone did not enable sufficient discrimination between fluorescence emission and reflected excitation light, resulting in detector saturation under all conditions tested. Had appropriate dichroic mirrors been available to enhance discrimination further, it is possible that these dyes would have exhibited a tractable signal-to-noise ratio.

### Dual Readout Assay

As several dyes demonstrated excellent separation between positive and negative controls during 3-day incubation, we considered them potentially useful in a real-time HTS format cytotoxicity assay. Their robust performance in opaque white plates that enhance luminescent assay signal was particularly intriguing. Therefore, we sought, as proof principle, to test whether a representative, fluorescent, DNA-binding dye could be used in combination with a luminescence-based readout to set up a screening assay for small molecules that block intracellular growth and therefore virulence of the bacterial pathogen, *L. pneumophila*.

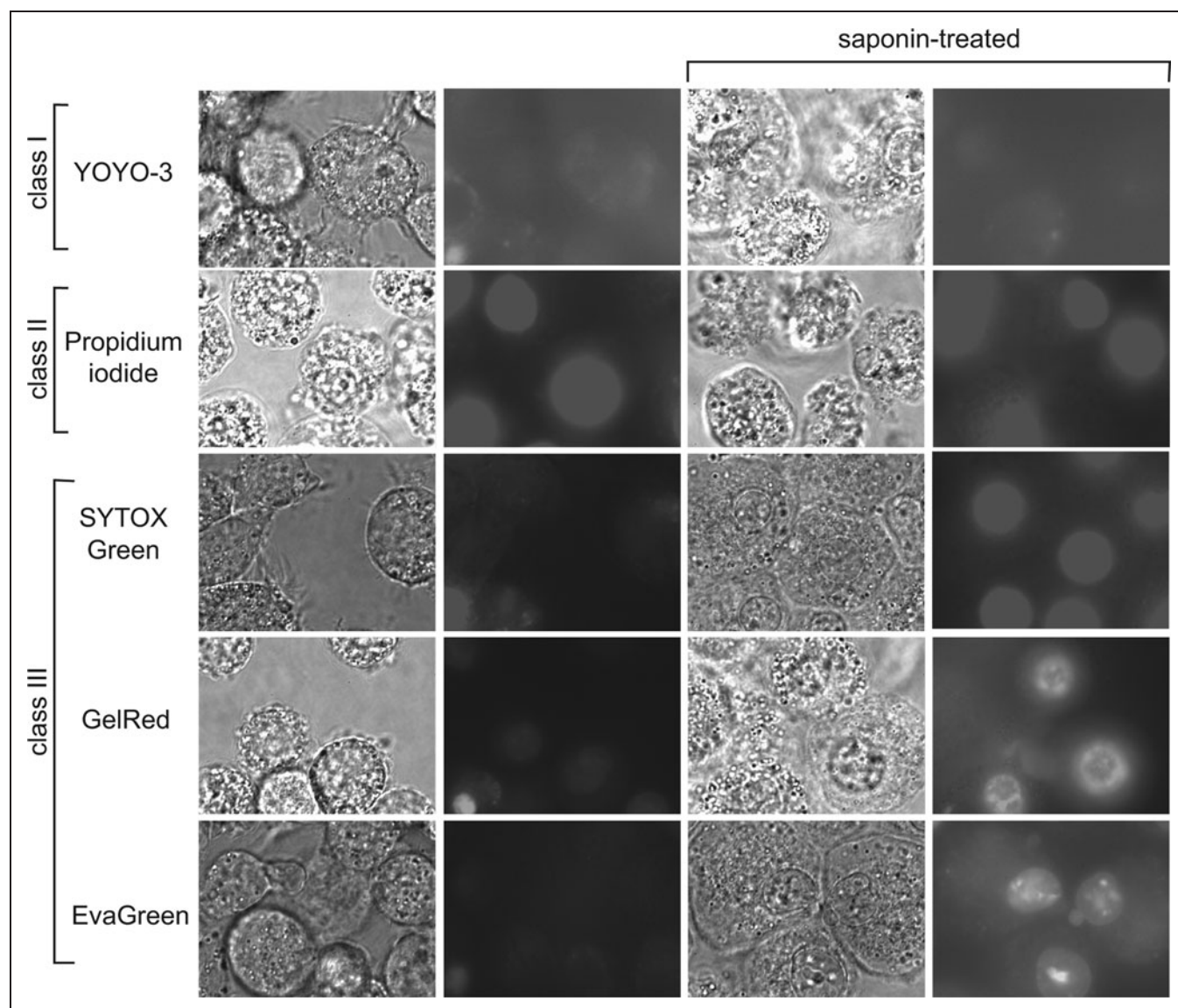
To allow us to quantify intracellular bacterial replication in HTS format, we made use of a previously described *L. pneumophila* strain expressing the *luxCDABE* operon from *P. luminescens*.<sup>10</sup> *Legionella* is noteworthy for its replication within macrophages, but inability to replicate in tissue culture medium.<sup>17</sup> Therefore, any increase in luminescence observed during macrophage infection with this strain should reflect intracellular replication. Furthermore, our own qPCR experiments (data not shown) and prior analysis<sup>10</sup> indicated that luminescence was an accurate quantitative indicator of intracellular bacterial growth. Notably, the bacterial *lux* operon encodes both luciferase and substrate.<sup>18</sup> Therefore, in contrast to mammalian luciferase systems, addition of exogenous substrate is not required prior to readout.

Several considerations are important for interpretation of data from a screen seeking to identify compounds that inhibit intracellular growth of *L. pneumophila*. Importantly, intracellular bacterial growth is dependent on host cell viability. Therefore, compounds that are cytotoxic to host cells will lead to a large class of biologically uninteresting “false positives”—in this case, low luminescence resulting from host cell toxicity, rather than inhibition of intracellular bacteria replication. In addition, bacterial replication itself is toxic to host macrophages at late time points.<sup>17</sup> Therefore, we expect true inhibitors of intracellular growth to reduce luminescence and prevent host cell death. A dual-assay readout for both luminescence and cytotoxicity would therefore prove advantageous in simultaneously identifying potential inhibitors of intracellular growth and ruling out a large class of false positives.

We therefore made use of observations with DNA-binding dyes to design an assay with dual readout for both bacterial growth (luminescence) and eukaryotic cell toxicity (fluorescence) in the same

**Table 3.  $Z'$  for Nonpermeable Dyes Over Time**

	Day 0	Day 1	Day 2	Day 3
SYTOX Green				
Black dish	ND	0.02	0.56	0.48
White dish	0.87	0.78	0.61	0.56
SYTOX Orange				
Black dish	0.25	0.52	0.48	0.21
White dish	0.64	0.51	0.41	0.21
CellTox Green				
Black dish	ND	0.36	0.49	0.48
White dish	0.79	0.58	0.79	0.84
EvaGreen				
Black dish	0.28	0.64	0.59	0.43
White dish	0.82	0.64	0.88	0.69
GelGreen				
Black dish	0.64	0.50	0.64	0.52
White dish	0.81	0.83	0.81	0.80
RedDot2				
Black dish	ND	−17	−3.1	−3.1
White dish	ND	0.65	0.44	0.39
ND, not determined.				

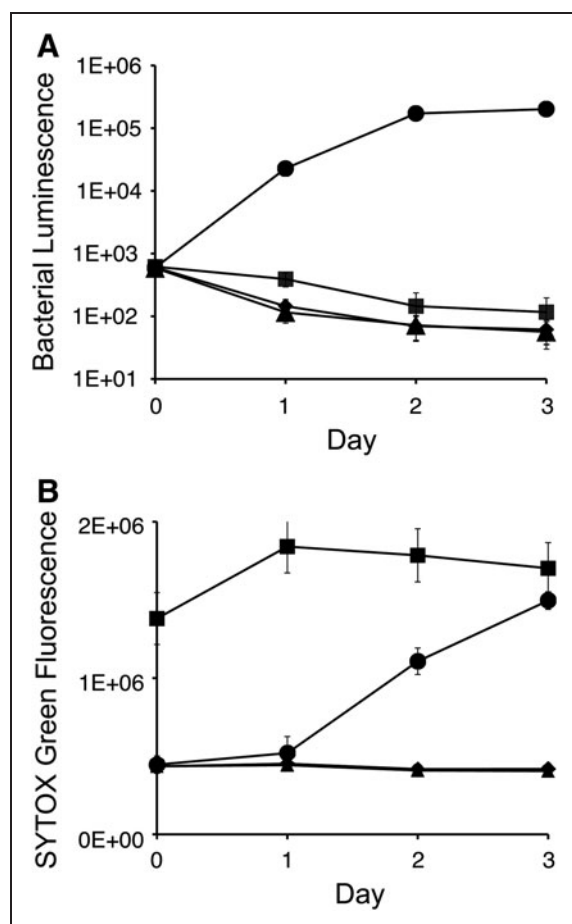


**Fig. 1.** Three classes of DNA-binding dyes. J774 cells were incubated with indicated dyes for 3 days, and scored by phase contrast and epifluorescence microscopy without or with treatment with saponin (following 3-day incubation) to permeabilize cells. Representative images are shown. Note that in the nonpermeabilized photomicrographs, an attempt was made to capture one spontaneously permeable, apoptotic cell nucleus in the lower left-hand corner as an internal reference point for fluorescence comparisons. Dyes were scored as cytoplasmic permeable, nontoxic (class I); permeable, toxic (class II); or nonpermeable, nontoxic (class III).

screening well. For proof of principle experiments, a cytotoxicity positive control (the permeabilizing agent, saponin) and two antibiotics highly active against intracellular *Legionella* growth (levofloxacin and azithromycin) were dissolved in DMSO and arrayed in a pin transfer plate. Robotic pin transfer to white 384-well screening plates containing J774 cells was then effected, followed by the combined addition of bacteria and SYTOX Green reagent. Plates were incubated and read daily for 3 days.

As shown in Figure 2, in DMSO-treated negative controls, bacterial luminescence increased roughly three logs during a typical experiment. At later time points (day 2–3), macrophage viability was re-

duced (increasing fluorescence) consistent with known lysis of host cells by replicating bacteria at later time points. As expected, antibiotics prevented both bacterial growth (increased luminescence) and associated host cell death (increased fluorescence). Lastly, a cytotoxic compound, saponin (specifically toxic to mammalian cells),<sup>14</sup> led to host cell death (high fluorescence) and thereby prevented intracellular growth (low luminescence). Saponin is not cytotoxic to bacteria because of absence of cholesterol in bacterial membranes,<sup>19</sup> therefore also likely accounting for modestly higher luminescence in saponin-treated relative to antibiotic-treated controls. Of note, bacterial luminescent signal in the DMSO negative control was robustly



**Fig. 2.** Intracellular bacterial growth and cytotoxicity measured in a dual readout high throughput screening (HTS) format assay. J774 cells were infected with *Legionella pneumophila* Lp02:flaA:luc in the presence of SYTOX Green. Luminescent (A) and fluorescent signals (B) of representative conditions: negative DMSO control (●); positive cytotoxicity control, saponin (■); and bacterial inhibition controls, azithromycin (◆) and levofloxacin (▲). Data points represent the mean and standard deviation of 192 replicates from a representative experiment with the exception that in the data shown for azithromycin luminescence, two outlier wells were excluded from analysis. As expected, bacterial luminescence (displayed in log scale) increases over time as a result of intracellular growth in macrophages. At later time points, bacterial growth kills host cells leading to increase in SYTOX Green fluorescence. We envision use of this dual readout assay to identify specific inhibitors of intracellular bacterial growth. The dual readout will allow us to easily identify and exclude false positive hits (e.g., saponin) that limit intracellular bacterial growth through destruction of the host cell (low luminescence, high fluorescence).

separated from positive antibiotic- and saponin-treated controls on days 2 to 3 ( $Z' = 0.5-0.7$ ), consistent with limitation of intracellular growth through inhibitory effects on bacteria or viability of the host cell (Table 4). Interestingly, on days 2 and 3, SYTOX Green fluorescent signal was significantly lower in antibiotic-treated positive controls (levofloxacin and azithromycin vs.

**Table 4.**  $Z'$  for Dual Real-Time Fluorescence and Luminescence HTS Readout

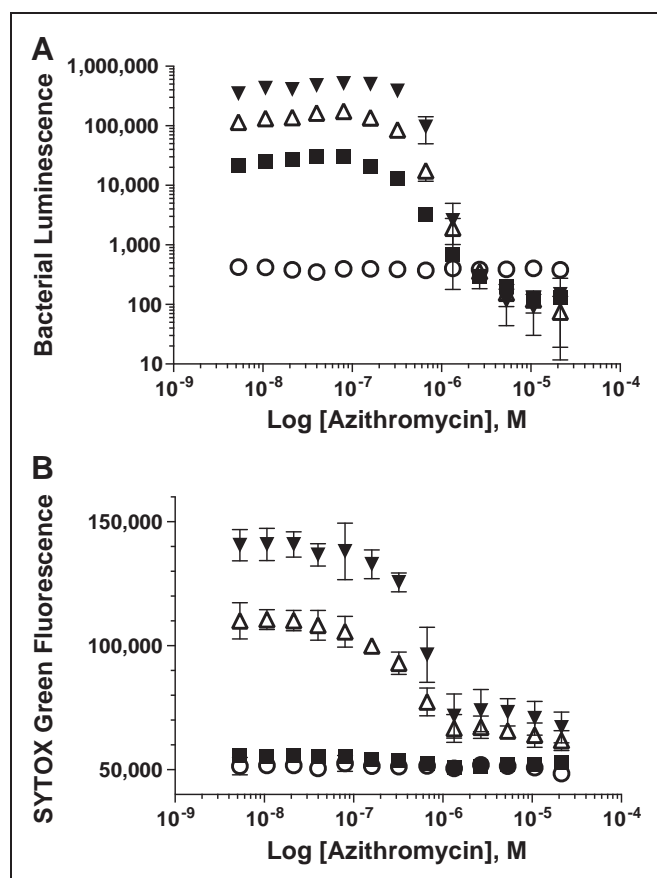
	Day 0	Day 1	Day 2	Day 3
Fluorescence				
Saponin vs. DMSO	0.29	0.38	-0.13	-2.20
Azithromycin vs. DMSO	-19.4	-4.69	0.53	0.78
Levofloxacin vs. DMSO	-19.3	-3.82	0.58	0.82
Saponin vs. levofloxacin	0.58	0.60	0.60	0.60
Luminescence				
Saponin vs. DMSO	-24.7	0.36	0.51	0.71
Azithromycin vs. DMSO	-37.9	0.35	0.51	0.71
Levofloxacin vs. DMSO	-67.4	0.38	0.51	0.71
Saponin vs. levofloxacin	-16.7	-0.47	-4.05	-4.35

DMSO;  $Z' = 0.5-0.7$ ), suggesting that compounds that block infection could also be identified simultaneously by their ability to prevent host cell death. Conversely, cytotoxic compounds could be identified by increased fluorescence signal on day 1 (saponin vs. DMSO;  $Z' = 0.4$ ).

These predictions were further supported by dose-response analysis performed for a representative antibiotic, azithromycin (Fig. 3). Here, the  $IC_{50}$  for luminescence and fluorescence were almost identical, and also similar to the minimal inhibitory concentration previously reported for macrophage grown *Legionella*.<sup>20,21</sup> Therefore, based on these similar dose-response profiles, bacterial replication, as assessed by luminescence, and cytotoxicity, as assessed by fluorescence, also appear linked.

As this dual real-time assay appeared to meet goals for identification of inhibitors of intracellular bacterial replication, we therefore set up a pilot screen with libraries containing 5,956 known bioactive compounds to examine performance of the assay during an actual HTS. The real-time assay afforded us the ability to read wells on both days 1 and 2 of incubation, allowing us to choose dish readings that provided the best statistical separation between positive and negative control wells in individual experiments (highest within plate  $Z'$ ). This was helpful in addressing the biological variability in this cell-based assay; for example, *Legionella* growth might peak earlier in some experiments than others.

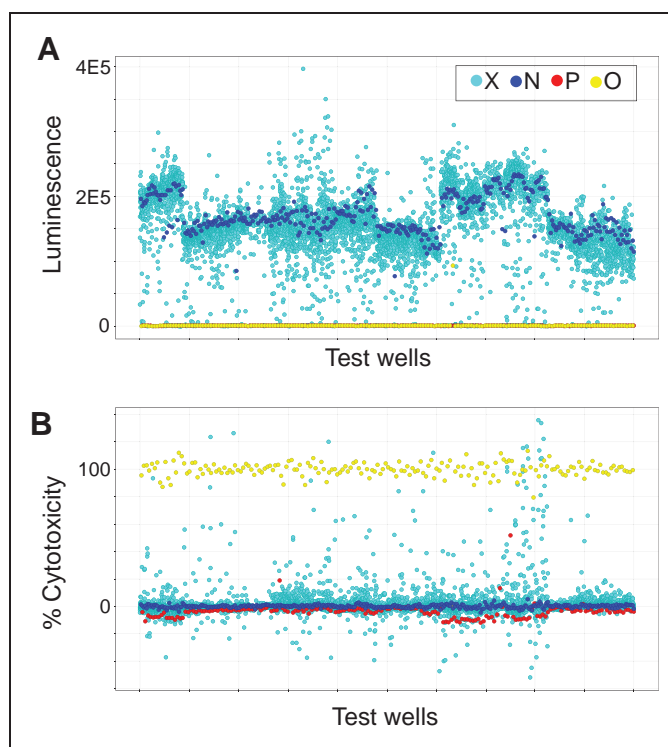
For luminescence, within plate  $Z'$  (levofloxacin vs. untreated controls) were generally slightly better for day 2 than day 1 readings (day 1 median plate  $Z'$  and interquartile range [IQR] of 0.67 and 0.15 respectively; day 2 median and IQR of 0.73 and 0.174 respectively). Day 2 luminescence  $z$  scores were therefore almost always used preferentially. For detecting compound cytotoxicity, day 1 fluorescence  $z$  score readings were generally used preferentially to help us best differentiate compounds that killed or protected J774 cells, prior



**Fig. 3.** Dose–response for azithromycin. Luminescence (A) and SYTOX Green fluorescent (B) signals on days 0 (○), 1 (■), 2 (△), and 3 (▼) after infection and treatment with twofold serial dilutions of antibiotic. Note that luminescence was plotted in log<sub>10</sub> scale to best illustrate the logarithmic growth pattern of *Legionella* and differentiate patterns of antibiotic effect. The data presented are the mean and standard deviation for 16 test wells per condition, 8 wells each from two separate 384 well plates. IC<sub>50</sub> for non-log transformed luminescence data on days 1, 2, and 3 were 189, 221, and 328 nM, respectively, and for fluorescence were 223, 303, and 407 nM, respectively.

to excessive bacterial replication-associated cytotoxicity that might mask direct toxic effects of compounds. Note, the convergence of saponin and DMSO control signal on day 2 versus day 1 in Figure 2B. This choice was also supported by within plate *Z'* determinations for saponin versus infected, untreated negative controls. Specifically, for fluorescence, *Z'* were almost always better for day one readings (day 1 median and IQR of 0.84 and 0.16; day 2 median and IQR of 0.53 and 1.3 respectively). Overall, *Z'* may have improved during this pilot bioactive screening compared to the HTS validation phase (Table 3) as a result of introduction of a room temperature equilibration period before each reading to reduce edge effect.

A summary of the screening results for day 2 luminescence and day 1 fluorescence data are shown in the scatterplots in Figure 4.



**Fig. 4.** Scatter plots for high throughput screening data. (A) Average luminescence signal from duplicate screening wells on day 2. (B) SYTOX Green fluorescence signal on day 1 normalized to mean negative control (0%) and mean saponin positive control (100%) values within each screening dish. Key: test compounds (X, cyan); negative controls (N, blue); levofloxacin, antibiotic positive controls (P, red); and saponin, cytotoxicity positive controls (O, yellow). Note, in (A), red, levofloxacin-positive control symbols are hidden behind yellow, saponin-positive control symbols and therefore not visible, as both demonstrated close to zero luminescence.

Controls performed as expected, specifically nearly complete suppression of luminescent signal in the presence of antibiotic or saponin, and high luminescent signal in negative controls (Fig. 4A). As predicted, fluorescent signal (percent normalized cytotoxicity) was high in saponin-treated controls. In contrast, test compound fluorescent signal was centered around the negative control (Fig. 4B and data not shown). In addition, antibiotic-treated controls demonstrated modestly lower cytotoxicity than negative controls, consistent with suppression of the very low level of cytotoxicity associated with limited bacterial growth on day 1 (Fig. 2B). Of note, screening was performed in duplicate in separate 384-well plates. There was excellent correlation between these replicates with an *R*<sup>2</sup> of 0.91 for luminescent *z* scores and an *R*<sup>2</sup> of 0.98 for fluorescent *z* scores (data not shown), suggesting high intra-assay reproducibility.

During the pilot HTS screen, we identified 316 strong screening hits defined by a luminescent *z* score >6.75 in both of the duplicate screening plates. We reasoned that true hits should in general demonstrate less cytotoxicity than the negative control (*i.e.*, a negative *z*



score). Therefore, a fluorescence  $z$  of  $\geq 1$  was set as a conservative threshold to identify false positive, eukaryotic cell cytotoxic hits. After excluding cytotoxic compounds in this way, 213 screening hits representing 135 unique compounds remained. These included 74 known antimicrobials that were represented often repeatedly in different bioactive libraries, the majority of which were traditional antibiotics in fluoroquinolone, tetracycline, sulfa, rifampin, and macrolide classes. There were also 61 other compounds that may have either unexpected antimicrobial activity or may act in some other way, whether on the host macrophage or bacterial cell, to limit intracellular infection. Cytotoxic compounds with a fluorescence  $z$  score  $\geq 1$  included those such as actinomycin D, gramicidin, dehydrocholic acid, mercurics, the blistering agent cantharidin, and chemotherapeutics such as idarubicin, camptothecin, and mitomycin that are presumed to limit intracellular bacterial growth through destruction of host cells. The use of a dual readout real-time assay allowed us to exclude these compounds as true hits based on eukaryotic cell toxicity. Therefore, during a pilot HTS, the dual real-time screen appeared to identify compounds with known eukaryotic cytotoxic and/or antimicrobial properties in accordance with our predictions.

## DISCUSSION

Membrane-impermeant, DNA-binding dyes have long been used in endpoint assays to ascertain eukaryotic cell membrane integrity and thereby serve as a marker for cell viability. Here, we explored whether such dyes could be used as real-time monitors of cell death through inclusion during the entire assay incubation. From a theoretical standpoint, this appeared practical if dyes remained nonpenetrant and nontoxic over prolonged periods. Indeed, the fluorescent DNA-binding dyes, CellTox Green and DraQ7, have either been promoted by their manufacturer or used in this fashion for microplate screening<sup>22</sup> and flow cytometry-based analysis<sup>23,24</sup> respectively. However, to the best of our knowledge, their performance in microplate format has not been evaluated in the peer-reviewed literature, nor compared to the large number of “impermeant” DNA-binding dyes that are commercially available.

Notably, a large number of impermeant fluorescent stains have been marketed for use in applications such as fluorescent microscopy, flow cytometry, agarose gel DNA staining, and real-time PCR detection. Of note, manufacturers have marketed some impermeant DNA-binding dyes as environmentally safe DNA stains, with the idea that impermeant dyes would less likely mutagenize living tissue and as such could therefore also be disposed of as nonhazardous waste. Several such “safe” dyes are now offered as alternatives to ethidium bromide and SYBR Green, used for agarose gel staining and real-time PCR assays respectively.<sup>25</sup> An observed utility in high throughput assay format would therefore also make these dyes welcome alternatives for the potentially large hazardous waste stream generated in HTS efforts.

We confirmed that the majority of impermeant DNA-binding dyes failed to penetrate a representative macrophage cell line during prolonged incubation. However, the dyes that performed well in microplate format generally had excitation and emission characteristics

similar to FITC. They maintained high  $Z'$  during a 3-day incubation (sustained statistical separation of saponin-treated and untreated J774 cells), consistent with low permeance. In contrast, both red and blue fluorescent dyes were unusable in microplate assays: use of the former limited by low signal-to-noise ratios, and use of the latter potentially limited by filters and dichroic mirrors available to us.

Notably, SYTOX Green, EvaGreen, and GelGreen all performed well in 384-well plate format, and are relatively inexpensive on a per assay basis. CellTox Green performed similarly but was significantly more expensive on a per well basis. We found that each gave good to excellent separation in white dishes ( $Z' > 0.5$ ) and good separation in black dishes ( $Z' > 0.4$ ) when comparing positive and negative controls during a 3-day incubation. Furthermore, in proof of principle HTS simulation experiments, dyes in this group were found to be amenable for use in dual readout assays in combination with a separate luminescence reporter (Fig. 2; data for other dyes not shown). Lastly, in a dual readout HTS screen of 5,956 known bioactive compounds, both known antimicrobials and cytotoxic compounds were identified according to prediction. Importantly, we did not note any interaction or interference of the real-time fluorescence and a bacterial luciferase assay whose output spanned a very large dynamic range (Figs. 2, 3, and 4A). Accordingly, we speculate that our findings should be applicable to use of fluorescent cytotoxicity detection in combination with other mammalian luciferase-based assays more commonly used in HTS readouts. Taken together, we believe that several membrane-impermeant DNA-binding dyes can serve as useful real-time monitors of eukaryotic cell viability, whether used alone or as a secondary readout, in HTS assays.

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## DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

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Address correspondence to:

James E. Kirby, MD  
Beth Israel Deaconess Medical Center  
330 Brookline Avenue—YA309  
Boston, MA 02215

E-mail: jekirby@bidmcharvard.edu

Abbreviations Used

- ATCC = American Type Culture Collection
- ATP = adenosine triphosphate
- CCD = charged coupled device
- DAPI = 4',6-diamidino-2-phenylindole
- DMSO = dimethyl sulfoxide
- DNA = deoxyribonucleic acid
- FITC = fluorescein isothiocyanate
- HTS = high throughput screening
- IC<sub>50</sub> = half maximal inhibitory concentration
- IQR = interquartile range
- ND = not determined
- PCR = polymerase chain reaction
- qPCR = quantitative polymerase chain reaction
- SD = standard deviation
- TRITC = tetramethylrhodamine