Application Note

Cell Analysis

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Immune Cell Killing Assays Using xCELLigence RTCA eSight: Optimizing Fluorescent Labeling Conditions and Image Acquisition Parameters

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Introduction

Using gold impedance biosensors embedded in the bottom of microplate wells, the Agilent xCELLigence RTCA line of instruments noninvasively monitor the health and behavior of cancer cells in real time. Because cellular impedance is a composite readout that reflects changes in cancer cell number, cell size, cell-substrate attachment strength, and cell-cell adhesion (i.e., barrier function), it is an extremely sensitive means of interrogating the efficacy of diverse cancer therapeutics, including engineered immune cells. Since immune cells are not adherent, they do not interact with the gold biosensors and, consequently, in an immune cell-mediated killing assay, the impedance signal exclusively reflects the health of the target cancer cells. Although this simple yet powerful approach is typically used as the primary analytical method, historically many xCELLigence users have corroborated the impedance data by temporarily removing the electronic plate (E-Plate) at key time points to collect photos. The newest xCELLigence instrument, the xCELLigence RTCA eSight, simplifies this workflow by coupling real-time impedance monitoring with live cell imaging, all in the same plate.

While eSight's brightfield images qualitatively demonstrate that the impedance signal is an accurate reflection of the killing process, overlap and clustering of target and effector cells (especially at late time points and high E:T ratios) confound quantitative analyses of these colorless images (Figure 1). Fluorescent labeling of the target cancer cells, the effector cells, or both makes it possible to quantify cancer cell killing from a perspective that is orthogonal to the impedance readout, while also shedding light on other facets of the killing process such as effector proliferation and the composition of large multicell clusters. While the Agilent panel of lentiviruses can be used to generate stable cell lines that express red, green, or blue fluorescent proteins, this approach may not be appropriate for some types of target cancer cells (such as primary tumor samples) or the engineered immune cells being studied. For this reason, alternative strategies are needed that enable each cell type to be

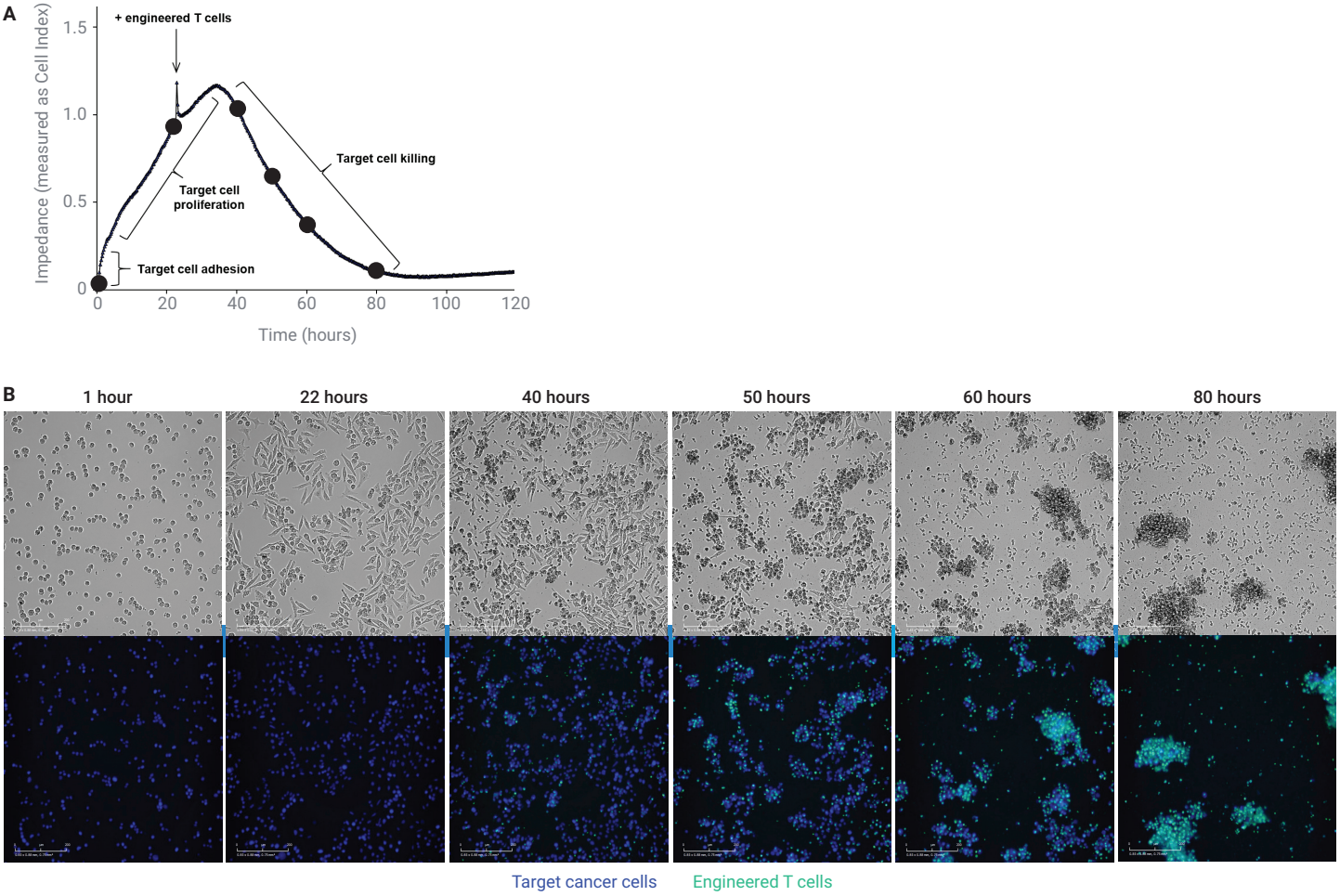

Figure 1. Brightfield images corroborate the eSight impedance data, but fluorescently labeling the cells makes the assay even more information rich. (A) Impedance trace showing the adhesion and proliferation of target cancer cells, which are killed upon addition of engineered T cells. Black dots correspond to the time points shown below in panel B. (B) Brightfield photos (upper panels) and dark-field + blue and green fluorescence photos (lower panels) for the time points highlighted in panel A. While the brightfield images correlate well with the impedance signal, overlap and clustering of the cells makes quantitative analysis of these images difficult. Labeling the effectors and targets different colors increases the amount of information that can be extracted, providing insight into cluster composition, effector cell proliferation, etc. Scale bars = 200 µm.

labeled with a unique fluorophore. This application note describes pulse labeling methods based on inorganic quantum dots, organic nanoparticles, dyes that covalently modify cytoplasmic proteins, and baculovirus. After evaluating each reagent's labeling efficiency across a population of cells, brightness within individual cells, and temporal persistence over the course of an assay, along with its spectral overlap and toxicity, we make recommendations for running fluorescence-based heterogeneous killing assays on eSight. A companion application note provides detailed recommendations for analyzing the fluorescence data generated using the above approaches (see "Immune cell killing assays using xCELLigence RTCA eSight: Analyzing fluorescent data").

Materials and methods

Cell maintenance and assays were conducted at 37 °C/5% CO_2. Cells, their source, and their growth medium are shown in Table 1. All FBS was heat inactivated (Corning, part number 35016CV). Immunocult media (part number 10981), IL-2 (part number 78145), and the Human CD3/CD28 T Cell Activator (part number 10971) used for stimulating T cell proliferation were from StemCell Technologies.

The different fluorescent labeling reagents used in this study are summarized in Table 2.

Unless otherwise specified, for all eSight assays, impedance was measured every 15 minutes, while images were acquired once per hour.

Table 1. Cells, source, and growth media.

Cell Line	Source	Growth Medium
A549	ATCC (part number CCL-185)	F-12K + 10% FBS
H1975	ATCC (part number CRL-5908)	RPMI 1640 + 10% FBS
HeLa	ATCC (part number CCL-2)	EMEM + 10% FBS
PC3	ATCC (part number CRL-1435)	F-12K + 10% FBS
T47D	ATCC (part number HTB-133)	RPMI 1640 + 10% FBS
HEK293A	Thermo Fisher Scientific (part number R70507)	DMEM + 10% FBS
MDCK	ATCC (part number CCL-34)	EMEM + 10% FBS
RPMI 8226	ATCC (part number CCL-155)	RPMI 1640 + 10% FBS
Primary T cells	Cellero (part number 1017-5063MA21)	Immunocult media + IL-2 (600 IU/mL) + CD3/CD28 T Cell Activator (30 µL/mL)
624.38 Mel	Gift from Michael Nishimura (Loyola U.)	DMEM + 10% FBS

Table 2. Fluorescent labeling reagents.

Pulse Labeling Reagent	Type of Fluorophore	Source
Qtracker 525	Inorganic nanoparticles	Thermo Fisher Scientific (part number Q25041MP)
Qtracker 625	Inorganic nanoparticles	Thermo Fisher Scientific (part number A10198)
Qtracker 655	Inorganic nanoparticles	Thermo Fisher Scientific (part number Q25021MP)
LuminiCell Tracker 540	Organic nanoparticles	Millipore Sigma (part number SCT010)
LuminiCell Tracker 670	Organic nanoparticles	Millipore Sigma (part number SCT011)
Incucyte Cytolight Rapid Green	Protein-reactive dye	Sartorius (part number 4705)
Incucyte Cytolight Rapid Red	Protein-reactive dye	Sartorius (part number 4706)
ViaFluor 405	Protein-reactive dye	Biotium (part number 30068)
ViaFluor 488	Protein-reactive dye	Biotium (part number 30086)
ViaFluor 650	Protein-reactive dye	Biotium (not yet sold publicly)
CellLight Nucleus-GFP, BacMan 2.0	Baculovirus-encoded GFP	Thermo Fisher Scientific (part number C10602)

Labeling suspended cells with Qtracker 655/625/525

1. To prepare a 30 nM labeling solution, 3 µL of Qtracker Component A was mixed with 3 µL of Component B in a 1.5 mL Eppendorf tube, followed by a 5 minute incubation at room temperature.
2. After adding 200 µL of complete growth medium to this mixture, it was vortexed for 30 seconds.
3. All 206 µL of this labeling solution was then mixed with a cell suspension consisting of 0.5 to 1.0 × 10^6 cells in ~100 µL of complete growth medium. The sample was then incubated at 37 °C for 2 hours.
4. Labeled cells were pelleted, supernatant was aspirated, and cells were resuspended in 600 µL of complete medium. This washing step was completed twice.
5. Cells were then seeded in an Agilent E-Plate View 96 at a density of 4,000 to 6,000 cells per well.

   Exposure times for Qtracker 655, 625, and 655: 300 ms

Labeling suspended cells with LuminiCell Tracker 670/540

1. A 10 nM labeling solution was prepared by directly diluting the stock of LuminiCell Tracker with complete growth medium that was prewarmed to 37 °C.
2. Cells were prepared by suspending 50,000 to 100,000 in 100 µL of complete growth medium that was prewarmed to 37 °C.
3. A 300 µL amount of the labeling solution and 100 µL of the cell suspension were mixed, followed by incubation at 37 °C for 2 hours.
4. Labeled cells were pelleted, supernatant was aspirated, and cells were resuspended in 600 µL of complete medium. This washing step was completed twice.
5. Resuspended cells were then seeded in an E-Plate View 96 at a density of 4,000 to 6,000 cells per well.

   Exposure times for LuminiCell Tracker 670 and 540: 300 ms

Labeling suspended cells with Cytolight Rapid Red/Green

1. A 100x labeling solution was prepared by diluting dye in PBS.
2. Two microliters of 100x dye solution was added to 200 µL PBS containing 1.0 × 10^6 cells, followed by thorough mixing.
3. Incubation was at 37 °C for 30 minutes.
4. A 1,200 µL amount of complete medium was added (to quench excess dye).
5. Cells were pelleted, supernatant was aspirated, and cells were resuspended in 600 µL of complete medium. This washing step was completed twice.
6. Cells were seeded in an E-Plate View 96 at a density of 4,000 to 6,000 cells per well.

   Exposure times for Cytolight Rapid Red and Green: 300 ms

Labeling suspended cells with ViaFluor 650/488/405

1. A 100x labeling solution was prepared by diluting the dye stock with DMSO.
2. Cells were suspended in prewarmed PBS at a density of 0.8 to 1.2 × 10^6 cells per mL.
3. The 100x labeling solution was combined with the cell suspension at a volumetric ratio of 1:99.
4. The reaction mixture was incubated at 37 °C for 30 minutes.
5. An equal volume of prewarmed complete culture medium was then added, followed by incubation at 37 °C for 5 minutes (to hydrolyze any free dye).
6. Labeled cells were pelleted, supernatant was aspirated, and cells were resuspended in an equal volume of complete medium.
7. Cells were incubated at 37 °C for an additional 15 to 30 minutes to allow ViaFluor dyes to react with intracellular proteins.
8. Cells were pelleted, supernatant was aspirated, and cells were resuspended in complete medium before seeding in E-Plate wells at a density of 4,000 to 6,000 cells per well.

   Exposure time for ViaFluor 650, 488, and 405: 300 ms, 300 ms, and 100 ms, respectively.

Labeling adhered cells with baculovirus

Unlike the other labeling reagents used in this application note, baculovirus particles are not inherently fluorescent, and therefore do not have the potential to produce a nonspecific fluorescent signal by adhering to the bottom of the well. Therefore, there are no obvious advantages to using baculovirus to transduce cells that are in suspension, before seeding them into E-Plate wells. Accordingly, the following protocol only addresses transduction of adherent cells.

1. On day one, cells were seeded in an E-Plate View 96 at a density of 4,000 to 6,000 cells per well.
2. On day two, 25 µL of BacMan reagent was added directly to wells (which already contained 150 µL of complete growth medium). Note that the inclusion of butyrate, which can sometimes improve transgene expression from baculovirus, was tested but was not found to be helpful in this context.

   Exposure time for baculovirus-encoded GFP: 300 ms

Results and discussion

Consideration of labeling strategies

When added to the assay growth medium, Agilent eLive Red and eLive Green dyes pass through the cell membrane and become ~1,000-fold more fluorescent upon binding to dsDNA. This simple, nonperturbing labeling strategy is useful in assays involving a single cell type (such as drug screening). However, in the context of an immune cell-mediated killing assay where two cell types are present, eLive dyes label all cell types the same color, making it impossible to extract information specific to the targets or effectors. For this reason, fluorescently labeling each cell type a different color before initiating the killing assay is advantageous. While transducing each cell type with a unique lentivirus (expressing RFP, GFP, or BFP) would meet this need, this approach is not always acceptable.* As an alternative to generating cell lines that stably express fluorescent proteins, effectors and targets can instead be transiently pulse labeled with different fluorophores.

Because it has an impact on how bright cells will be throughout the course of a killing assay, an important parameter to consider for pulse labeling strategies is the time at which cells are labeled. Pulse labeling delivers a fixed number of fluorophores into the original population of cells. Each time one of these cells divides, the two progeny cells both receive roughly half the number of dye molecules that were present in the parent, causing the fluorescent intensity of individual cells to decrease proportionally over time. With this in

* Reasons for this include: 1) Some cell types transduce inefficiently, 2) the extra time required for transduction and subsequent selection of transductants might not be tolerable, and 3) introducing a fluorescent protein encoding gene into the genome of immune cells that are destined for patient transfusion might be unacceptable.

mind, one may opt to seed target cells, allow them to attach and proliferate overnight, and then pulse label them in their adhered state immediately before effectors are added. While this helps to ensure maximal brightness of the targets during the killing process, a disadvantage is that trace amounts of the fluorescent dye can sometimes bind directly to the well bottom, thereby increasing the background signal (Figure 2A. Note that this photo was taken immediately after labeling target cells but prior to effector cell addition). The extent to which this is a problem varies depending on the identity of the dye, the material of the well bottom (plastic versus glass), and the rigor of washing steps post labeling. If high background is indeed found to be a problem one can instead label the target cells while they are still in suspension (post trypsinization), wash away excess dye, and then seed the labeled cells into E-Plate wells (Figure 2B). After allowing sufficient time for the labeled target cells to adhere, effector cells can be added. This "labeling in suspension" strategy was used in all studies presented in this application note.

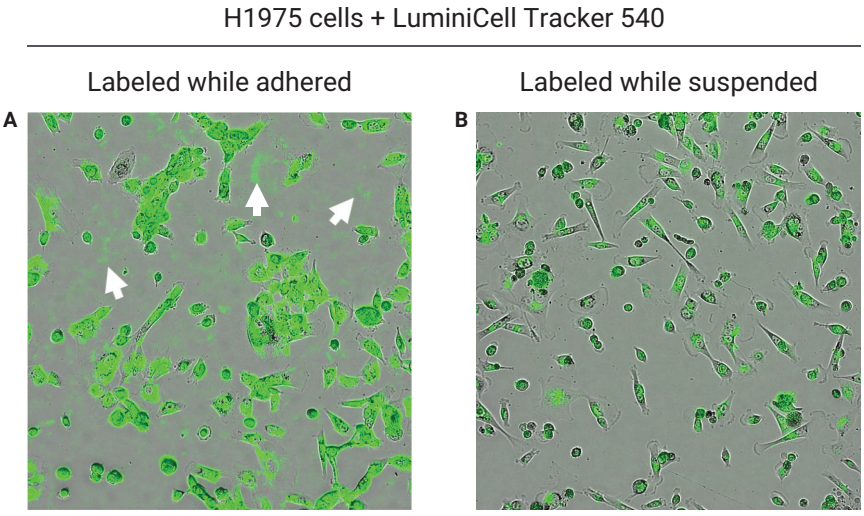

Figure 2. The time at which target cells are pulse labeled can impact both their brightness during the killing assay, and the level of background signal. (A) 5,000 H1975 cells were seeded in an Agilent E-Plate well. 24 hours later, the adhered cells were labeled with 2.5 µM LuminiCell Tracker 540 for 2 hours. After two wash steps, cells were re-immersed in complete growth medium. Photo was taken immediately thereafter. The three white arrows highlight regions where dye has nonspecifically bound to the well bottom (i.e., there are no cells in these regions). (B) Immediately after being harvested by trypsinization, H1975 cells suspended in complete growth medium were labeled with 2.5 µM LuminiCell Tracker 540 for two hours. Cells were subsequently pelleted, washed twice, and were then resuspended in complete growth medium and seeded into an E-Plate well. This photograph was taken 24 hours after labeling/seeding (consequently, the cells in panels A and B had both been adhered for a similar amount of time when the photos were taken). Nonspecific binding of the dye to the well bottom is not observed under this condition.

Evaluating pulse labeling efficacy

Aiming to assess a broad range of pulse labeling strategies, this study used 10 different fluorescent reagents that operate by four different mechanisms of action. After labeling cells in suspension using the manufacturer's recommended protocol, excess fluorophore was washed away, cells were seeded into E-Plates at a density of 4,000 to 6,000 cells/well, and photos were collected 24 hours later. This process was repeated with eight different cell types to interrogate each reagent's breadth of use.

The Qtracker reagents consist of inorganic nanocrystals that are coated with a peptide that promotes endocytosis. Upon uptake, these nanocrystals persist within endocytic vesicles throughout the cytoplasm. Although eight different versions of Qtracker are available, where the size of the nanocrystals determines their excitation and emission wavelengths, the two that are most compatible with the eSight optics are Qtracker 625 and 655. Figure 3A demonstrates that 24 hours after being labeled with Qtracker 655, seven of the cell lines display intense and uniform red fluorescence. In contrast, T cells label less efficiently – with only ~30% of the cells displaying fluorescence. Similar results were also observed for Qtracker 625.

Although they also label cells via an endocytic mechanism, LuminiCell Tracker reagents differ from Qtracker reagents in that they are nanoparticles comprised of aggregated organic fluorophores. As shown in Figure 3A, both the red and the green versions of LuminiCell Tracker label all eight of the cell lines tested, but with variable efficiency and brightness. For example, when using LuminiCell Tracker 540 only a small percentage of RPMI 8226 cells are actually fluorescent. When using LuminiCell Tracker 670, the majority of MDCK cells display fluorescence, but the brightness is quite low. Similar to what was observed with the Qtracker reagents, only a fraction of T cells labeled with LuminiCell Tracker reagents actually display fluorescence.

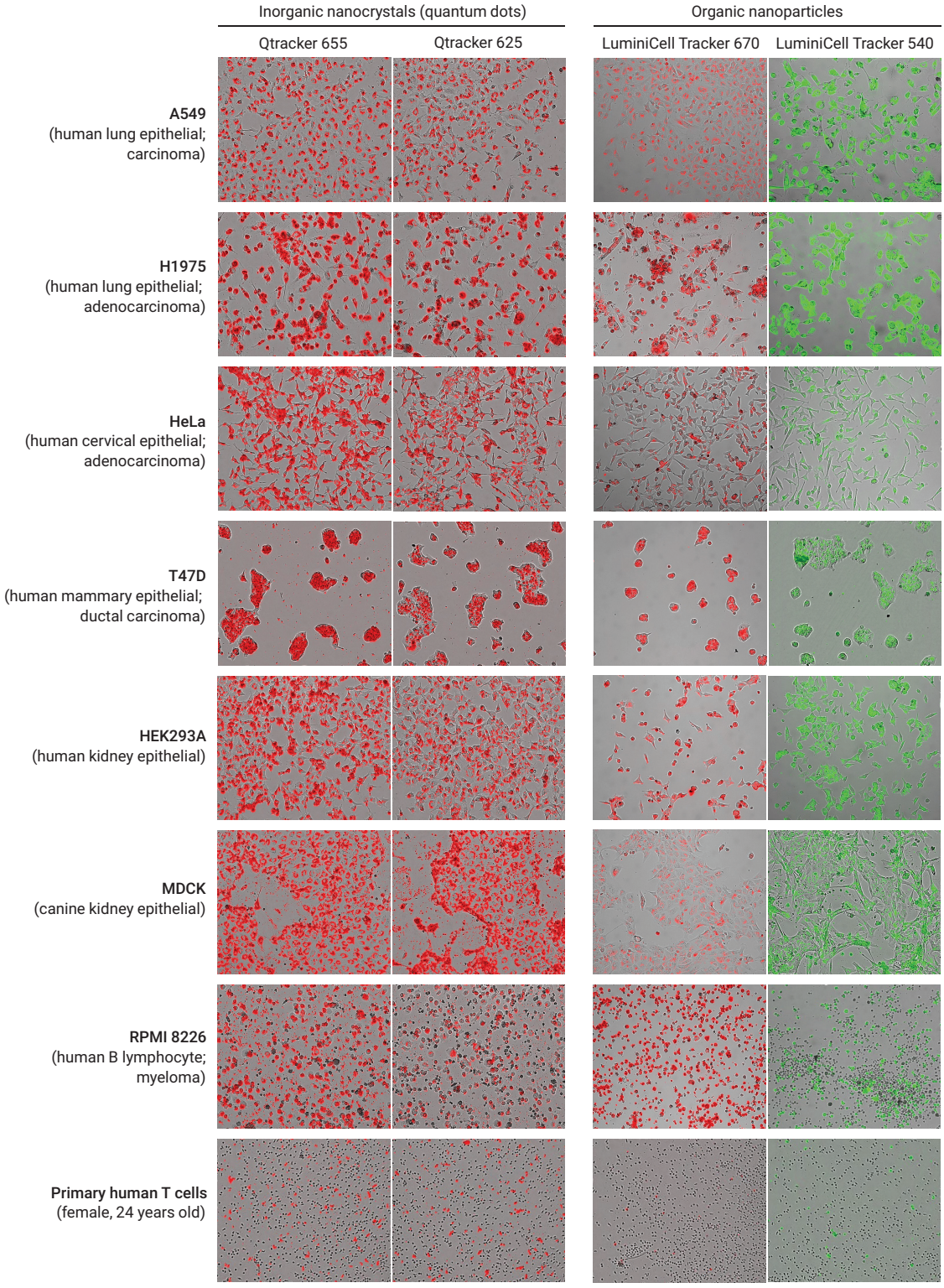

Figure 3A. Qualitative evaluation of nanoparticle-based pulse labeling reagents in eight different cell types. Photos for a given cell line sometimes look substantially different depending on which reagent they have been labeled with. This can be a consequence of the unique way each fluorescent reagent localizes within cells.

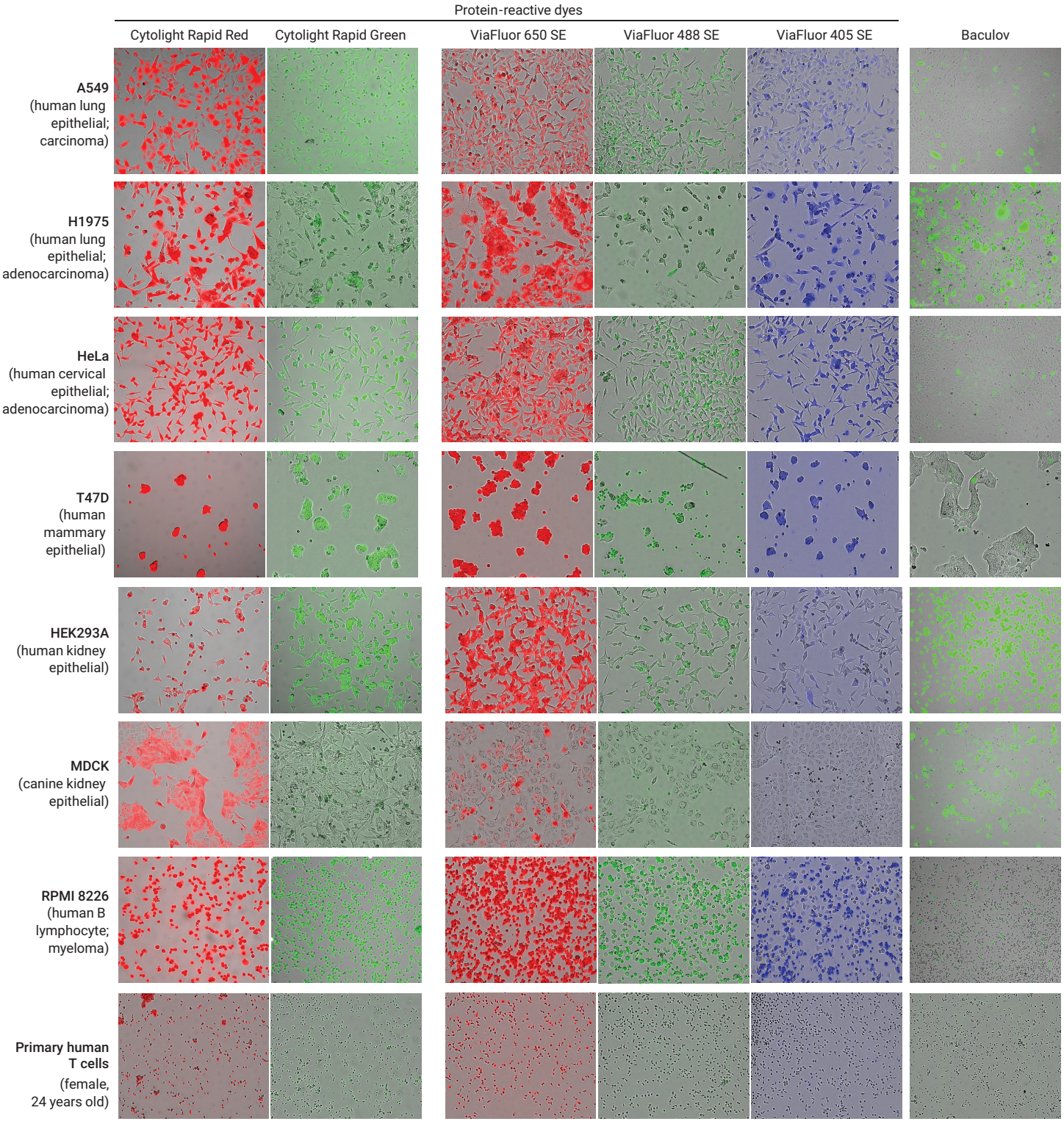

Figure 3B. Qualitative evaluation of small molecule- and baculovirus-based pulse labeling reagents in eight different cell types. Photos for a given cell line sometimes look substantially different depending on which reagent they have been labeled with. This can be a consequence of the unique way each fluorescent reagent localizes within cells.

Labeling cells by a completely different mechanism of action, the three different ViaFluor dyes initially exist as membrane permeable esters. Once these dye molecules enter the cytoplasm, nonspecific esterases cleave away an aliphatic chain to generate a molecule that 1) is charged and therefore cannot diffuse back through the cell membrane, and 2) is reactive with the primary amine groups of proteins. This results in the fluorophore being covalently linked to cytoplasmic proteins. As shown in Figure 3B, the red and green ViaFluor reagents are capable of efficiently labeling most of the cell lines; MDCK cells are the exception. The blue ViaFluor reagent displays greater cell type-dependency, with some cell types labeling extremely well (H1975, T47D) and other cell types labeling poorly (MDCK and HEK293A). Operating by a mechanism that is similar to the ViaFluor dyes, the Cytolight Rapid Red/Green dyes label all eight of the cell lines with efficiencies that are roughly similar to the red and green ViaFluor dyes (Figure 3B).

As one last alternative, labeling with baculovirus, was also evaluated. In contrast to lentivirus which integrates into the host cell genome, baculovirus simply delivers its genome to the host cell nucleus – whereupon it can autonomously direct synthesis of a fluorescent protein. In contrast to the nanoparticles and small organic dyes described previously, which endow cells with fluorescence very quickly, expression of fluorescent protein (GFP) from the baculovirus genome takes approximately 10 hours. Among the eight cell types tested, baculovirus was found to generate a fluorescent signal with highly variable efficacy (Figure 3B).

Although the images in Figures 3A and 3B provide a glimpse of how a given labeling reagent performs in diverse cell types, it is important to recognize that the fluorescent brightness of cells can be modulated by varying both the labeling conditions and the image acquisition parameters. More importantly, getting cells to fluoresce is relatively easy; achieving fluorescence in a manner that is not perturbing to the biology being studied is more challenging – as addressed in the following section.

Balancing brightness and toxicity via labeling conditions

A key lesson from Figure 3 is that the brightness of a labeling reagent can vary substantially between different cell lines. While these differences might reflect the efficiency of endocytosis (for nanoparticles such as Qtracker and LuminiCell Tracker), the relative activities of esterases and efflux pumps (for amine-reactive dyes such as Cytolight Rapid and ViaFluor), or transduction efficiency and suppression of exogenous gene expression (for baculovirus), no effort was made to probe these possibilities mechanistically. In situations where increased fluorescent intensity is desired, increasing the dye concentration and the duration of the labeling reaction can be helpful. Figure 4A shows the impact of titrating the concentration of Qtracker 655 in H1975 cells, while leaving the duration of the labeling reaction constant (2 hours). Increasing the fluorophore concentration increases both the percentage of cells that are labeled and the brightness of individual cells.

Even though high fluorescent intensity is generally regarded as advantageous (both for aesthetics and for quantitative analysis), one must always balance brightness with toxicity. Though numerous vendors sell reagents that are marketed as being compatible with live cell imaging, many of these will still perturb cell health and behavior if used at high concentrations. This is illustrated in Figure 4B where impedance traces reveal that in H1975 cells Qtracker 655 is nonperturbing at 10 and 30 nM, but displays toxicity at 90 nM. Using eSight's brightfield channel and plotting the total brightfield area as a function of time also reveals toxicity at 90 nM (Figure 4C), although the effect is less substantial than that observed by impedance.

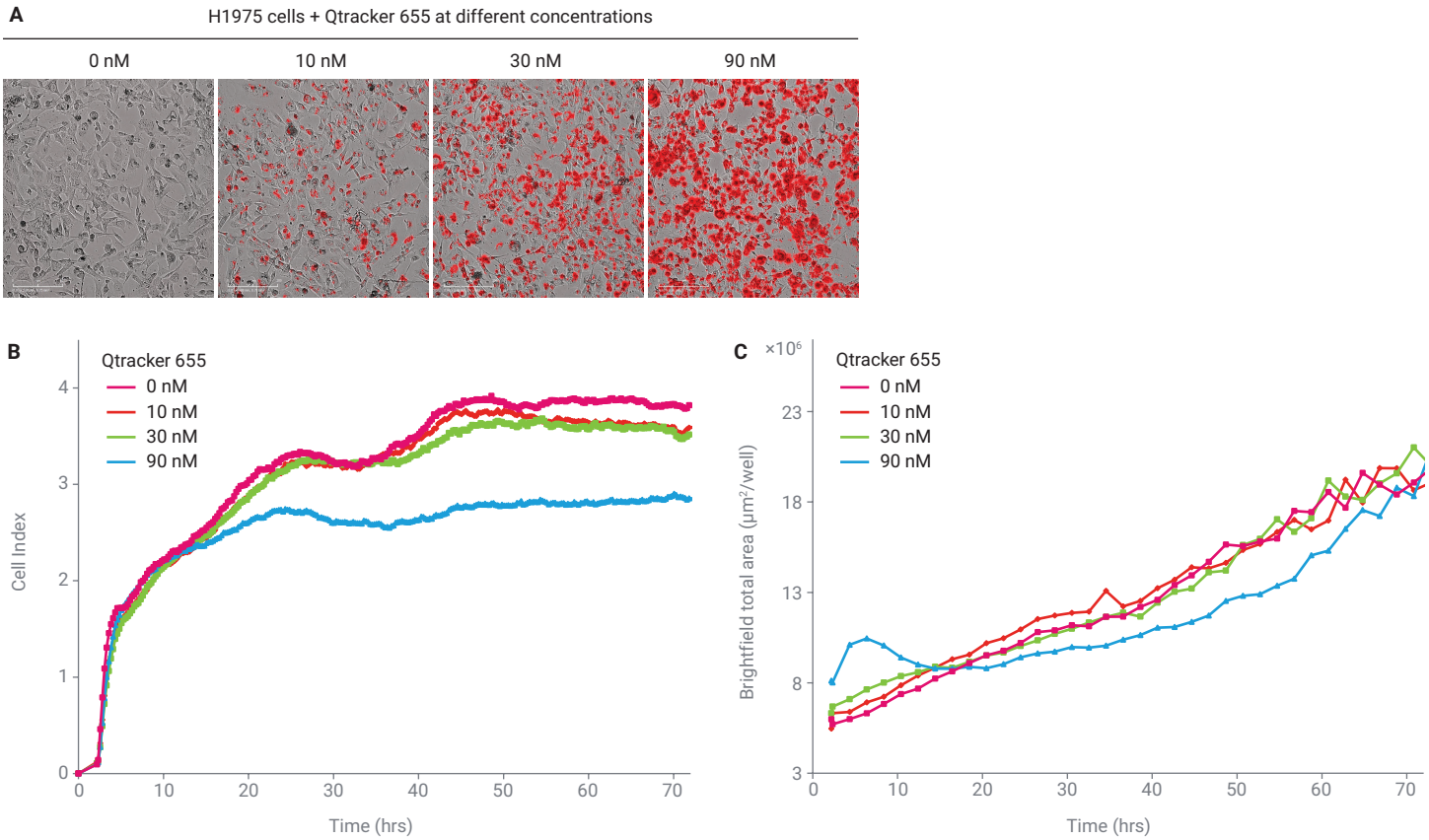

Figure 4. Balancing brightness with toxicity. (A) When H1975 cells are pulse labeled with Qtracker 655 for two hours, both the percentage of cells that are labeled and the brightness of individual cells increase with increasing dye concentration. (B) Real-time impedance monitoring indicates that while 10 and 30 nM Qtracker 655 are nonperturbing, increasing the concentration to 90 nM alters cell health/behavior. (C) Monitoring the total brightfield area in real-time also indicates that 90 nM Qtracker 655 causes cells to behave differently than the unlabeled control.

Balancing brightness and toxicity via image acquisition parameters

Beyond labeling conditions, an additional parameter that can be adjusted to modulate fluorescent intensity is the duration of exposure (i.e., the length of time that cells are being illuminated and their emitted light is being collected). Working with H1975 cells that were pulse labeled with 1.5 µM ViaFluor 488 for 30 minutes, Figure 5A illustrates how varying exposure duration impacts the brightness of both the cells and the background. The functional impact of these different exposure durations was evaluated using impedance (Figure 5B). The red trace represents wells in which no photos were taken (neither brightfield nor green fluorescence) and therefore establishes a baseline for normal/healthy cells. When compared to this baseline, it is clear that longer exposure durations result in toxicity. While cells imaged for 50 and 200 ms behave similarly to the negative control, cells imaged for 400, 800, or 1,600 ms display a progressive drop in the impedance signal. This exposure-dependent toxicity was confirmed by photos showing altered cell morphology at later time points (not shown), and is clearly evident in plots of percent brightfield confluence (Figure 5C). At the 65 hour time point the confluence of ViaFluor 488 labeled cells was 78% (50 ms exposure), 64% (400 ms exposure), and 23% (1,600 ms exposure). Note that unlabeled H1975 cells also display exposure-dependent toxicity (gray traces in Figure 5C), but this toxicity is exacerbated by the presence of the ViaFluor 488 dye.

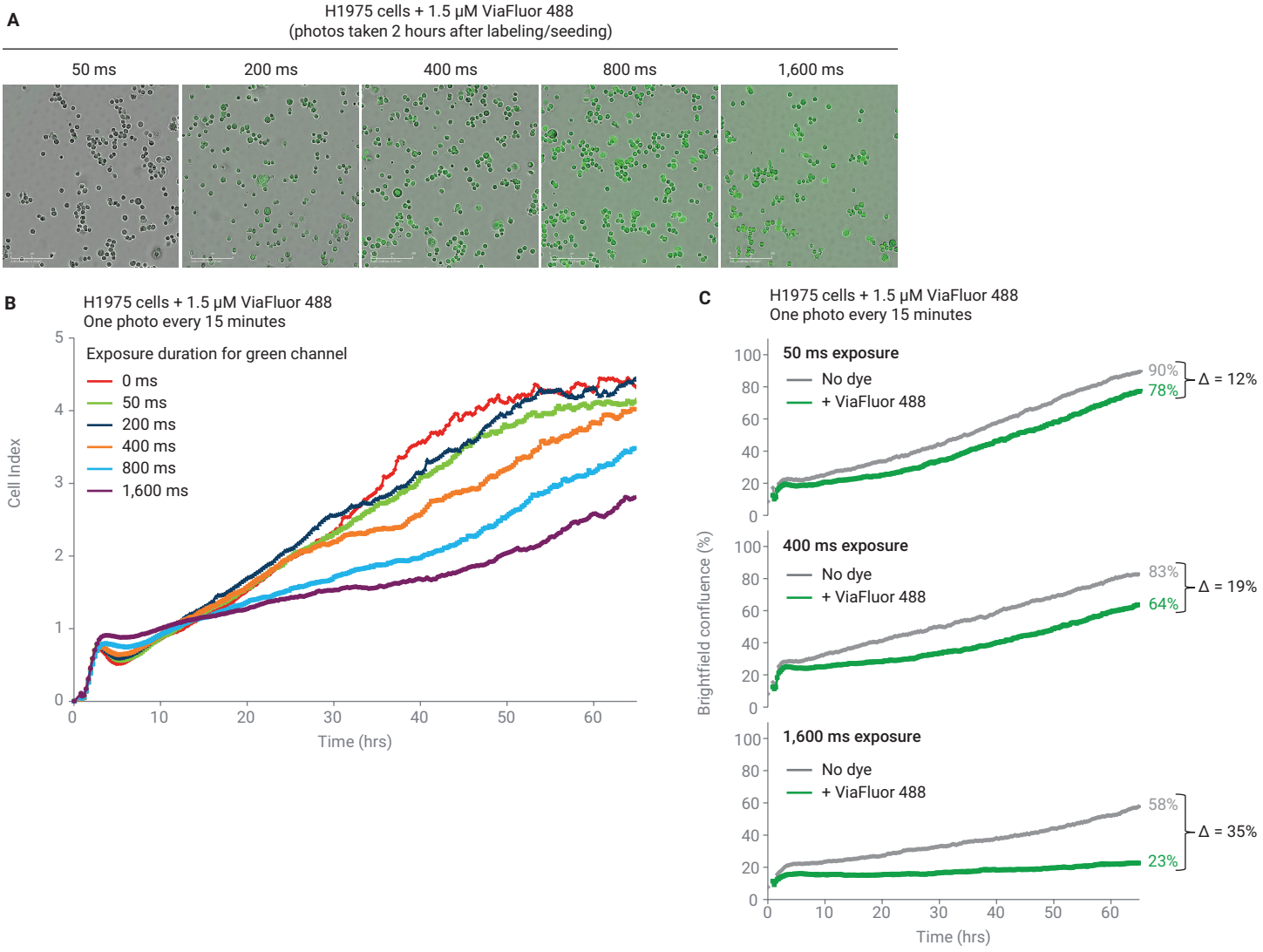

Figure 5. Phototoxicity as a function of exposure duration. (A) Photos of H1975 cells taken 2 hours after being labeled (in suspension) with 1.5 mM ViaFluor 488. (B) When these cells are imaged every 15 minutes, real-time impedance traces demonstrate the correlation between toxicity and exposure duration. (C) When unlabeled cells are subjected to green fluorescent image acquisition every 15 minutes, they display a progressive drop in percent brightfield confluence as a function of exposure duration (grey traces). A similar trend is observed in cells labeled with ViaFluor 488, but the exposure-dependent toxicity is more severe than what is observed in unlabeled cells.

All of the previously described behaviors reflect the well-characterized principles of phototoxicity, which is always a risk when live cells are subjected to fluorescent imaging. Endogenous molecules such as porphyrins and flavins absorb the wavelengths of light used in these types of assays. Once they have been pushed into an excited electronic state these molecules can react with oxygen, causing them to degrade while simultaneously producing reactive oxygen species (ROS).[1-3] ROS such as hydroxyl and superoxide radicals can subsequently damage DNA, proteins, lipids, and enzyme cofactors[4] – all of which contribute to declining cell health. Very importantly, the exogenous dyes used for fluorescent labeling can themselves contribute to ROS production[5], which is consistent with the ViaFluor 488-induced photosensitization shown in Figure 5C.

To minimize phototoxicity, the temporal frequency at which fluorescent images are collected can be reduced. Figure 6 compares the impedance traces of H1975 cells, again labeled with 1.5 µM ViaFluor 488, when they are imaged every 7.5, 15, 30, 60, 120, or 240 minutes. As a control, these cells were imaged using brightfield alone (Figure 6A); regardless of how frequently photos were collected, the impedance traces remained superimposable. In contrast, collecting green fluorescent images with an exposure of 800 ms causes the impedance signal to decrease in a manner that is proportional to the photo acquisition frequency (Figure 6B). While the traces for 240, 120, and 60-minute acquisition frequencies are similar, collecting photos every 30, 15, or 7.5 minutes causes the impedance to drop progressively. When the exposure duration is increased to 1,600 ms, this trend becomes even more noticeable (Figure 6C).

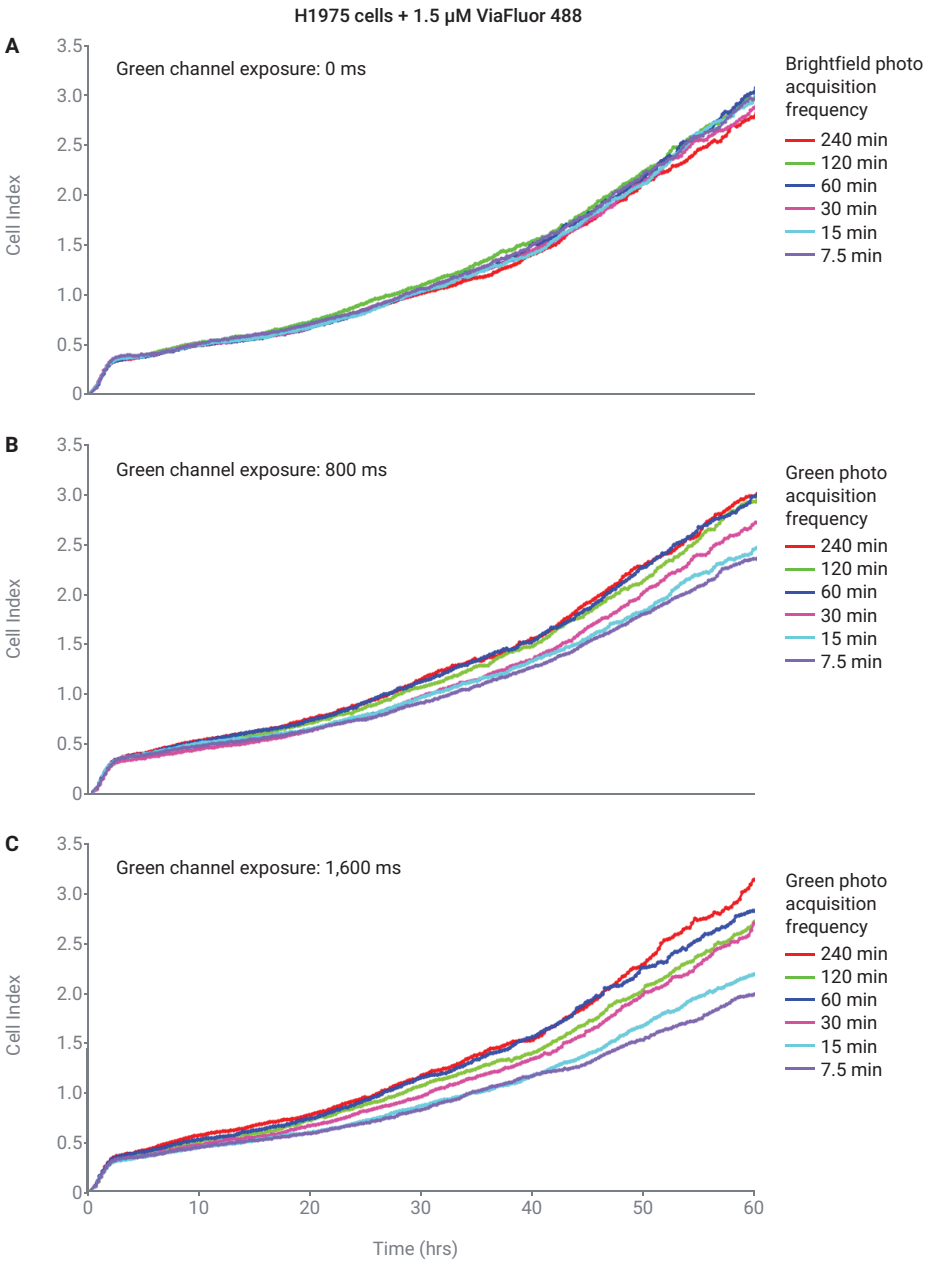

Figure 6. Phototoxicity can be minimized by collecting fluorescent images less frequently. H1975 cells labeled with 1.5 µM ViaFluor 488 were imaged with brightfield alone (A), brightfield + green fluorescence for 800 ms (B), or brightfield + green fluorescence for 1,600 ms (C). Under all three conditions, images were collected at different temporal frequencies: 240, 120, 60, 30, 15, or 7.5 minutes.

Note that the phototoxicity trends demonstrated above are a general phenomenon, but the specifics will vary for every unique combination of cell type, fluorescent dye, and excitation channel. For example, MDCK cells were found to be much more resistant to phototoxicity than the H1975 cells shown above. As another example of this, when testing ViaFluor 650 (red), ViaFluor 488 (green), and ViaFluor 405 (blue) dyes in parallel, H1975 cells displayed toxicity in the following order: blue > green > red (data not shown). This trend is to be expected considering the fact that the shorter wavelengths of light needed to stimulate blue florescence are more capable of causing cellular damage than the wavelengths of light that are needed to stimulate green or red fluorescence.

Dye combinations and spectral overlap

When running an immune cell-mediated killing assay, if one wishes for the effectors and targets to be different colors, care must be taken to ensure that the two dyes are spectrally compatible with one another. Specifically, one must confirm that each dye only fluoresces in a single channel. To do this, a single cell type is labeled with a particular dye and is then imaged in all three of eSight's fluorescent channels: red, green, and blue. Using this approach, it is clear that the small organic dyes Cytolight Rapid and ViaFluor only fluoresce within the single desired fluorescent channel (Figure 7). For example, Cytolight Rapid Red fluoresces in the red channel but not the green and blue channels. In contrast, LuminiCell Tracker 670, Qtracker 655, and Qtracker 625 were found to fluoresce in all three of eSight's fluorescent channels.* Consequently, if one of these dyes is used to label target cells the effector cells in that well would need to be unlabeled. Qtracker 525 and LuminiCell Tracker 540 were found to be slightly better in that they fluoresced in the blue and green channels but not the red channel. Thus, if target cells were pulse labeled with either of these dyes, effector cells could be labeled with a red dye.

When pulse labeling using baculovirus, the proteins mKate, eGFP, and tagBFP are recommended as they only fluoresce in a single eSight channel (not shown). Numerous other fluorescent proteins are also compatible with eSight's optics, but should be validated using the approach shown in Figure 7.

Temporal persistence of fluorescence

When evaluating different fluorescent labeling strategies, an important parameter to consider is the length of time over which the fluorescent signal remains usable. As mentioned in an earlier section, a challenge with the pulse labeling approach is that cells that are initially bright become progressively more faint with each round of cell division. In the top row of images in Figure 8, H1975 cells that have been pulse labeled with LuminiCell Tracker 540 are initially uniformly bright. However, by the 100 hour time point many of the cells display significantly reduced fluorescence. This trend is even more pronounced when H1975 cells are labeled with ViaFluor 488 (Figure 8, bottom row). By the 100-hour time point, most of the cells are no longer fluorescent.

The time window over which a given reagent will produce a usable fluorescent signal will vary based on the labeling conditions (concentration used, duration of labeling reaction) and the identity of the cell line. When designing and optimizing a labeling strategy, the goal is for the majority of the cells to still be fluorescent at the latest time point being analyzed. However, during the data analysis step there are ways to correct for the intrinsic fading of fluorescence that often occurs in pulse labeled cells. For a detailed description of these methods, see the companion application note "Immune cell killing assays using xCELLigence RTCA eSight: Analyzing fluorescent data".

* This is a consequence of the following: both organic and inorganic nanoparticles are excited by an extremely broad range of wavelengths of light.

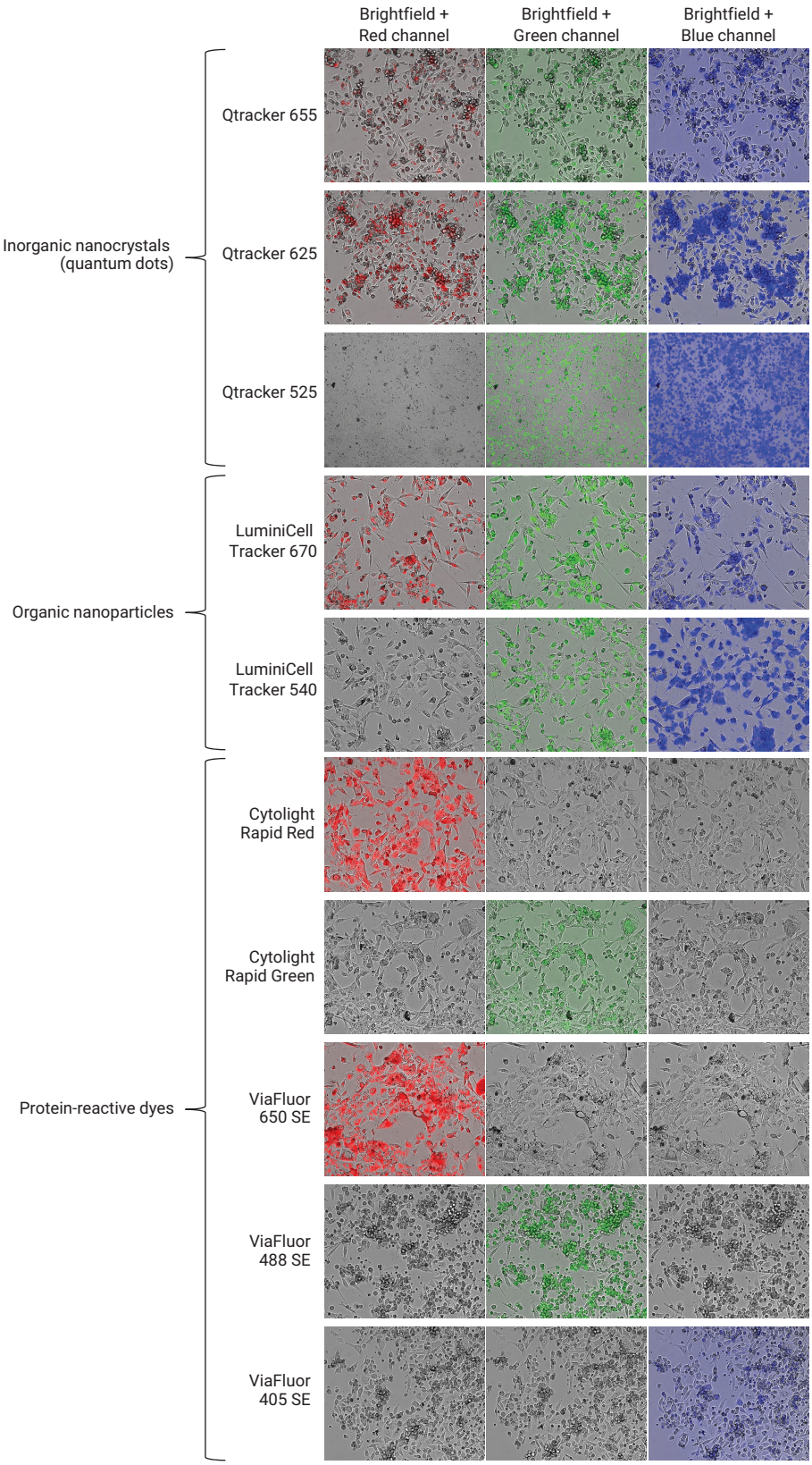

Figure 7. Evaluation of spectral overlap for 10 pulse labeling reagents. See text for details.

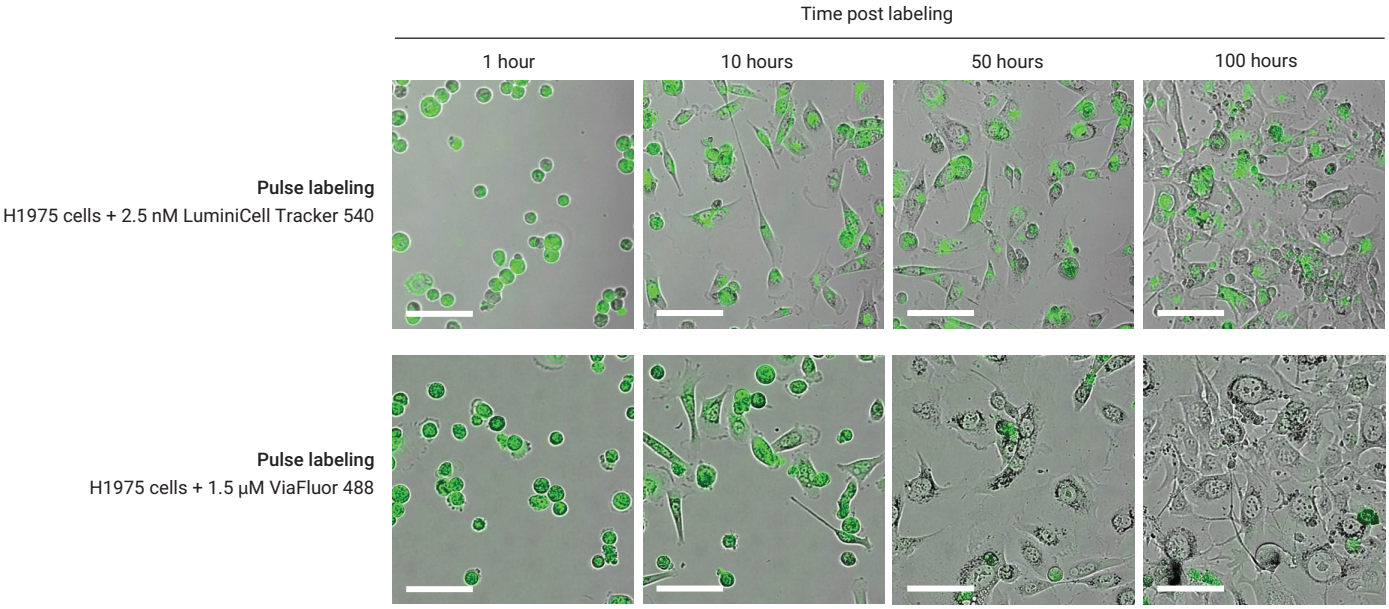

Figure 8. Temporal persistence of fluorescence. See text for details. Scale bars = 100 µm.

Conclusion

The ability to track cancer cell killing in real time without the need for labels makes cellular impedance an extremely powerful yet simple tool for studying the potency of engineered immune cells. Concurrently capturing brightfield images from the same well provides an orthogonal perspective that qualitatively corroborates the impedance signal (for the cell biologist, seeing is believing). However, because the effector and target cells tend to overlap and cluster, it is impossible to extract from these brightfield images quantitative information that is specific to each cell type. Labeling targets, effectors, or targets and effectors with unique fluorophores provides an image-based means of quantifying target cell death and effector cell proliferation that supplements the eSight impedance data. If the production of cell lines that stably express a fluorescent protein is not an option, pulse labeling can be a useful alternative as long as it is used judiciously.

Whereas cellular impedance monitoring has been shown, over 20 years and across many hundreds of cell lines, to be nonperturbing, live cell imaging has the potential to be much more problematic. The data presented in this study demonstrate that while it is fairly easy to find a combination of labeling conditions and imaging parameters under which cells can be made to fluoresce, a significant percentage of these combinations perturb cell health over the course of an assay. Therefore, fluorescent imaging is an art of compromise – where maximal fluorescent brightness and maximal temporal persistence of fluorescence must be balanced with minimal toxicity. This balancing act is unique neither to eSight nor the labeling reagents examined here: it exists for all live cell imagers and all fluorescent dyes.

As a road map to help eSight users get up and running with assays that use fluorescent pulse labeling, the following list summarizes the parameters to pay attention to, and the order in which to address them:

1. Fluorophore selection: Chose fluorescent dyes whose excitation and emission spectra are compatible with eSight's optics:

Channel	Excitation wavelength (nm)	Emission wavelength (nm)
Red	588 to 596	612 to 680
Green	473 to 491	503 to 561
Blue	383 to 401	430 to 462

Similar to selecting dyes for a multicolor flow cytometry panel, the dyes used in an eSight assay should have excitation and emission peaks that are as close as possible to the excitation and emission windows of eSight's bandpass filters. Exact matching is not necessary.

Because shorter wavelengths of light tend to be more phototoxic than longer wavelengths, red fluorophores should be used preferentially over green fluorophores, and green fluorophores should be used preferentially over blue fluorophores. Note that this is merely a suggestion based on first principles; many cell lines can indeed be imaged with a variety of blue fluorophores without showing any signs of toxicity.

To aid fluorophore selection, refer to the performance summaries provided in Table 3.

Table 3. Summary of fluorophore performance in the context of pulse labeling.

Reagent	Label Type	Maximum Brightness of Signal[1]	Persistence of Signal Over Time[2]	Spectral Overlap[3]	Quality of Labeling Efficiency Across Diverse Cell Lines[4]	Vendor and Catalog No.
Qtracker 525	Inorganic nanocrystals	+++	+++	Green Blue	Consistent	Thermo Fisher Scientific (cat. no. Q25041MP)
Qtracker 625	Inorganic nanocrystals	+++	+++	Red Green Blue	Consistent	Thermo Fisher Scientific (cat. no. A10198)
Qtracker 655	Inorganic nanocrystals	+++	+++	Red Green Blue	Consistent	Thermo Fisher Scientific (cat. no. Q25021MP)
LuminiCell Tracker 540 Green	Organic nanoparticles	+++	+++	Green Blue	Slightly variable	Millipore Sigma (cat. no. SCT010)
LuminiCell Tracker 670 Red	Organic nanoparticles	+++	+++	Red Green Blue	Slightly variable	Millipore Sigma (cat. no. SCT011)
Incucyte Cytolight Rapid Green	Protein-reactive dye	++	++	No	Slightly variable	Sartorius (cat. no. 4705)
Incucyte Cytolight Rapid Red	Protein-reactive dye	++	++	No	Consistent	Sartorius (cat. no. 4706)
ViaFluor 405 Blue	Protein-reactive dye	++	+	No	Variable	Biotium (cat. no. 30068)
ViaFluor 488 Green	Protein-reactive dye	+	+	No	Variable	Biotium (cat. no. 30086)
ViaFluor 650 Red	Protein-reactive dye	++	+	No	Slightly variable	Biotium (not yet sold publicly)
CellLight Nucleus-GFP, BacMan 2.0	Baculovirus	++	++	No	Highly variable	Thermo Fisher Scientific (cat. no. C10602)

[1] Maximum brightness: +++ = very bright; + = dim

[2] Persistence of signal over time: +++ = fluorescent signal remains bright for multiple days; + = fluorescent signal fades rapidly

[3] Spectral overlap: Does the reagent fluoresce in multiple eSight channels? If yes, which ones does it fluoresce in?

[4] Quality of labeling efficiency across diverse cell lines: Consistent = most cell lines display a similar degree of labeling; variable = the degree of labeling fluctuates dramatically across different cell lines.

2. Labeling conditions: Start off by labeling cells using the dye manufacturer's recommended labeling conditions. That being said, titrating both the dye concentration and the duration of the labeling reaction are highly recommended. This allows one to quickly sample a matrix of conditions all in a single assay.

3. Initial image acquisition parameters: As a starting point, collect photos using the settings outlined in Table 4.

A few hours after cells have been seeded and have begun to attach, use the Image Browser tab of the eSight software to determine whether the fluorescent images that are being acquired are of sufficient brightness. If they are too bright (i.e., saturated), use the Schedule tab to terminate the currently running step, add a new step wherein the exposure duration is reduced, and then start running the new step. If fluorescent images are too faint, follow this same process but increase the exposure duration. It is important to adjust the exposure duration settings early in the assay; if one waits to do this until late in the assay, it will be impossible to evaluate the accumulative impact of phototoxicity (as described in step 4).

4. Evaluating phototoxicity: When labeling conditions and image acquisition parameters are first being optimized, one should evaluate whether phototoxicity is an issue using impedance plots and, when possible, percent brightfield confluence plots. Using this two-pronged approach is essential: in some instances the impedance readout is more sensitive to toxicity than the brightfield readout, and in other instances the reverse is true. To detect and characterize phototoxicity, use the controls shown in Table 5. If phototoxicity is detected, it can often be minimized, if not eliminated, using the principles described in this application note.

Table 4. Recommended photo acquisition settings to start off with.

Parameter	Suggested Setting	Rationale
Temporal Frequency of Photo Acquisition	Once every 2 hours	For most killing assays, collecting photos every 2 hours provides sufficient resolution of the killing process without inducing phototoxicity.
Number of Fields of View Per Well	4	In a 96-well plate, eSight can collect 1 to 4 fields of view (i.e., images) from each well. Since cells occasionally distribute across a well bottom with non-uniform density, collecting four fields of view helps ensure that the data being collected are reflective of the well as a whole. If phototoxicity is found to be a problem, consider reducing this to two fields of view per well to minimize the total light exposure.
Exposure Duration	Brightfield: this is automatically optimized by the eSight Red: 400 ms Green: 400 ms Blue: 80 ms	For most pulse labeling dyes and fluorescent proteins (RFP, GFP, and BFP), the values shown here yield acceptable brightness without causing phototoxicity.

Table 5. Controls used to evaluate phototoxicity.

Control No.	Condition	Rationale
Control 1	No dye No brightfield imaging No fluorescent imaging	This serves as the "gold standard" for how healthy/non-perturbed cells behave.
Control 2	With dye With brightfield imaging With fluorescent imaging	Determines whether the combination of dye and excitation results in toxicity.
Compare the impedance data from Controls 1 and 2. If this comparison indicates that toxicity is an issue, set up a new assay to probe whether this is due to the particular dye that is being used versus the cell line being intrinsically sensitive to phototoxicity. If the latter is the culprit, switching to a different dye will not solve the problem. To help differentiate between these possibilities, run the same Controls 1 and 2 from above alongside Controls 3 and 4 shown below.		
Control 3	No dye With brightfield imaging With fluorescent imaging	Determines whether the cell line is intrinsically sensitive to phototoxicity.
Control 4	With dye No brightfield imaging No fluorescent imaging	Determines whether the dye molecule itself is toxic even when it is not being excited.

5. Suggested labeling scheme: All things considered, a preferred labeling scheme for an immune cell mediated killing assay is as follows. Pulse label target cells with LuminiCellTracker 540. Most target cells are labeled efficiently by this reagent, and its fluorescence persists even through very late time points. Although LuminiCellTracker 540 fluoresces in both eSight's blue and green channels, using the green channel is recommended as this uses longer wavelength (i.e., less energetic) excitation light. Concurrently, pulse label the immune cells with ViaFluor 650 (red). This combination of red and green fluorescence produces images which are both visually striking and easy to analyze.

For examples of immune cell killing assays that use the different labeling strategies presented here, and for a detailed description of how to best analyze the associated fluorescent data, please see the companion application note "Immune cell killing assays using xCELLigence RTCA eSight: Analyzing fluorescent data".

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