

Real time visualization of cancer cell death, survival and proliferation using fluorochrome-transfected cells in an IncuCyte[®] imaging system

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Abbreviations used: ATCC, American Type Culture Collection; CMV, cytomegalovirus; DEVD, caspase-3/7 recognition and cleavage sequence; EF1 α , elongation factor-1 alpha; MOI, multiplicity of infection; NEB, New England Biolabs; NK, natural killer; NLS, nuclear localization signal; PB, peripheral blood; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline

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ABSTRACT

Cancer immunotherapy is a rapidly advancing and viable approach to treating cancer along with more traditional forms of therapy. Real-time cell analysis technologies that examine the dynamic interactions between cancer cells and the cells of the immune system are becoming more important for assessment of novel therapeutics. In this report, we use the IncuCyte[®] imaging system to study the killing potential of various immune cells on cancer cell lines. The IncuCyte[®] system tracks living cells, labeled by a red fluorescent protein, and cell death, as indicated by the caspase-3/7 reagent, which generates a green fluorescent signal upon activation of apoptotic pathways. Despite the power of this approach, obtaining commercially fluorescent cancer cell lines is expensive and limited in the range of cell lines that are available. To overcome this barrier, we developed an inexpensive method using a lentiviral construct expressing nuclear localized mKate2 red fluorescent protein to stably label cancer cells. We demonstrate that this method is effective in labeling a wide variety of cell lines, allowing for analyses of different cancers as well as different cell lines of the same type of cancer.

Keywords: cancer, fluorescence, transduction

INTRODUCTION

The relationship between cancer and the immune system has been intensely studied for decades [1]. To use the intrinsic ability of the human immune system to recognize and eliminate tumors is an appealing therapeutic strategy [2,3]. However, cancer cells employ a number of immunosuppressant mechanisms that result in an ineffective immune response [4,5]. Multiple approaches in cancer immunotherapy (such as monoclonal antibodies, cancer vaccines and immune checkpoint therapy) are rapidly advancing the field, but challenges still exist in making these therapies effective [1,3,6].

The interaction between cancer cells and immune effector cells is complex and incompletely understood. Live-cell imaging technologies allow for visualization, characterization and measurement of biological processes in living cells. These technologies will play an important role in the development and validation of the next-generation of cancer immunotherapies. Simple live-cell imaging assays from target cell

apoptosis, cytotoxic cell polarization and cell migration assays to more complex co-culture systems, such as immune cell killing and phagocytosis assays, will be critical for the characterization of these functions and development of new therapeutics.

In the past 20 years, development of novel fluorescent tools to measure apoptosis by live-cell imaging technologies has facilitated the study of these cell processes and cell interactions [7]. These include the fluorogenic caspase substrates Phi-PhiLux and NucView488, which are non-toxic, cell-permeable reagents designed to track caspase activation in living cells [8-10]. These substrates consist of dyes that are linked to a caspase-3/7 recognition and cleavage sequence (DEVD) [11,12]. The substrates are not fluorescent until cleaved by caspase [7]. These fluorescent tools enable real-time detection and quantification of intracellular caspase-3/7 activity over hundreds of time points due to immunotherapy manipulation using live-cell imaging technologies. This technology offers a significant advantage over the other existing methods such as *in situ* nick-end labeling (TUNEL) assay, mitochondrial

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membrane potential assay, and annexin V and propidium iodide combination staining. These assays are limited in the number of time points that can be assayed, are time consuming to run, can require significant optimization to get reproducible data and often need to be coupled with a second assay to confirm a positive apoptotic result.

To further understand cancer cell-immune response dynamics, we fluorescently labeled multiple cancer cell lines to better visualize the immune cell interaction with cancer cells. The cancer cells were stably labeled using a lentivirus expressing nuclear localized mKate2 fluorescent protein (red). The lentiviral approach enables the establishment of stably fluorescent cancer cell lines in a rapid and cost-efficient manner. In these experiments, mKate2 (red) cancer cell lines were treated with IncuCyte® caspase-3/7 apoptosis reagent, a version of NucView488 (green), to measure apoptosis induced by immunotherapy treatments as visualized on the IncuCyte® Imager (Sartorius, USA). In this paper, we describe the methodology for generating fluorescent-labeled cancer cell lines for live-cell analysis on an IncuCyte® Imager.

MATERIALS AND METHODS

Lentiviral construction

Generation of the mKate 2X nuclear localization signal (NLS) lentiviral expression vector was done as follows. mKate cDNA was amplified from pmKate2-C vector (Evrogen) using the following primers: mKate F SphI 5'-AAT GCA TGC GCC ACC ATG GTG AGC GAG CTG ATT AAG GAG -3'; mKate 2X NLS R BamHI 5'- TAG AGG ATC CTT ACT TCT ACC TTT CTC TTC TTT TTT GGA TCT ACC TTT CTC TTC TTT TTT GGA TCA GCT CGA GAT CTT CCT CTG TGC CCC AGT TTG CTA GGG AGG -3'. The NLS sequence is underlined in the mKate 2X NLS primer above. PCR amplification of mKate 2X NLS was done using Phusion Taq Polymerase with the 5X GC Buffer (NEB) following the manufacturer's instruction using touchdown PCR cycling conditions [13]. The cycling conditions were as follows: 98°C 30 s 1 cycle; 98°C 15 s, 67°C (-0.5°C/cycle), 72°C 30 s 12 cycles; 98°C 15 s, 61°C, 72°C 30 s 61 cycles. The resulting mKate 2X NLS PCR product was isolated using the Monarch DNA Gel Extraction Kit (NEB), digested with SphI and BamHI, and ligated using the same sites in pLentiLox EF1 α -CMV-Puro lentiviral transfer vector (available from University of Michigan Vector Core) generating pLentiLox EF1 α -mKate 2X NLS-Puro. The vector was verified by Sanger sequencing. See **Fig. S1** for the full plasmid map, sequence, and primer design for pLentiLox EF1 α -mKate 2X NLS-Puro.

Lentiviral production

For lentivirus production, the packaging vectors psPAX2 (35 μ g), pC1-VSVG (35 μ g) and 70 μ g of pLentiLox EF1 α -mKate 2X NLS-Puro transfer plasmid were incubated with 420 μ g PEI (molecular weight 2500, Polysciences, Inc) in 10 ml of Optimem (Life Technologies) at room temperature for 20 min. Ninety milliliters of complete DMEM [(Gibco, Cat. #11965; 10% FBS (Hyclone) and 1 \times GlutaMAX (Gibco))] was added to the transfection mix and was distributed equally between 5-T150 flasks (Falcon) of 80% confluent HEK293T cells. Supernatants were collected and pooled after 72 h, filtered with a 0.45 micron HV-Durapore Stericup (Millipore), pelleted by centrifugation at 13000 rpm on a Beckman Avanti J-E centrifuge at 4°C for 4 h, and re-suspended at 10 \times the original concentration ($\sim 1 \times 10^7$ TU/ml) in DMEM (Gibco).

The lentivirus was stored in aliquots at -80°C.

Lentiviral transduction and cell isolation

One day prior to lentiviral transduction, cancer cells were seeded at 3.0×10^5 cells/well on 6 well plates (Corning) in media containing RPMI 1640 (HyClone), 10% FBS (HyClone), and 1% antibiotic-antimycotic solution (Gibco). Cell media was changed to 1.35 ml of fresh antibiotic-free RPMI 1640 with 10% FBS supplemented with 150 μ l of 10 \times virus (~ 6 MOI) and 4 μ g/ml Polybrene (Sigma-Aldrich). The cells were incubated at 37°C with 5% CO₂ for 24 h. Following incubation, cells were trypsinized (0.25%-EDTA, HyClone), collected, and centrifuged at 600 RCF for 6 min at 4°C in an Eppendorf 5702 R centrifuge. The cells were plated in RPMI 1640 with 10% FBS and 1% antibiotic-antimycotic solution on a T75 flask (Corning) and grown until confluent. Cells were trypsinized, collected and centrifuged as done previously. Following centrifugation, cells were re-suspended in 1 \times PBS (HyClone) and counted with a hemocytometer using Trypan blue (Life Technologies) exclusion. Cells were then centrifuged and re-suspended in Zombie Violet (BioLegend) diluted in 1 \times PBS (1:4000) at a concentration of $\sim 1 \times 10^6$ cells/ml and incubated on ice, in the dark, for 20 min. Cells were washed once with 1 \times PBS and then re-suspended at a final concentration of $\sim 2 \times 10^6$ cells/ml in 2% FBS and 2 mM EDTA (Invitrogen). The cells were then singly sorted into individual wells of a 96 well plate (Corning) containing RPMI 1640, 10% FBS, and 1% antibiotic-anti-mycotic solution at the University of Michigan Flow Cytometry Core using a Synergy Head Cell Sorter (Sony). The cells selected were negative for Zombie Violet [450/50 (405)] and in the top 5% of mKate fluorescence intensity [615/30 (561)]. The sorted cells were then incubated at 37°C with 5% CO₂ and monitored on the IncuCyte® Live Cell Analysis System (Sartorius, USA). Cell isolates with strong fluorescence and growth were selected for expansion.

Immune/cancer cell co-culture experiments

Peripheral blood mononuclear cells (PBMCs) used in co-culture experiments were activated, plated and grown similarly as described below unless stated otherwise. MDA 231 human breast cancer cells (HBCCs) were plated in a 96-well plate with a seeding density of 20000 cells per well. Non-activated (NA) or OKT3 activated PBMCs (50000 cells per well) were added to the MDA 231 cells ($n = 3$ wells for each cell line) at about 22 h. Cell killing was measured by evaluating the number of MDA 231 cells present in each well expressing caspase-3/7 reagent and results pooled. Co-culture conditions, including cell numbers and OKT3 activation of PBMCs were modified from Essen Instruments protocol "IncuCyte® Immune Cell Killing, Clustering & Proliferation Assay" which can be obtained online (https://www.essenbioscience.com/media/uploads/files/Immune_Cell_Killing-Clustering-Proliferation_Assays_brochure.pdf). Briefly, for OKT3 activated PBMCs, 1×10^6 cells were incubated with 10 μ g/ml OKT3 antibody overnight. Activated PBMCs were then counted and added to the co-culture as described above.

Isolation of human PB and lymphocytes

Peripheral blood (PB) from normal adult donors was collected in heparinized tubes. After centrifugation, the buffy coat was collected and mononuclear cells were purified under sterile conditions on an Accu-Prep gradient at 400 \times g for 30 min at room temperature. Mononuclear cells collected at the interface were washed twice with PBS and re-suspended in purified PBS (Life Technologies—Bethesda, MD). Mononuclear cell

viability was typically 98% as determined by trypan blue exclusion. Lymphocyte separation is performed using EasySep™ Human Cell Isolation Kits per the manufacturer's protocol (STEMCELL Technologies—Vancouver, BC). To prevent unwanted lymphocyte activation, lymphocyte subsets are isolated using a column-free magnetic negative isolation system that provided a reliable source of specific lymphocyte subsets for use in our assays. Some cells were fluorescently tagged for use in our cell imager. Cytospin examinations were performed upon all isolations to ensure purity of the cells used in our assays, and subset purity was routinely confirmed by FACS and/or cytopsin differential analysis.

Statistics

All data is expressed as the mean \pm standard error of the mean (SEM). All *in vitro* assays were evaluated with an unpaired, parametric Student's *t* test for each time point measured in the IncuCyte® cell imaging system. Two-tailed *P*-values < 0.05 were considered statistically significant.

RESULTS

Utilizing the IncuCyte® live-cell analysis system to quantify immune cell killing responses of cancer cell lines in real-time

We used the Sartorius IncuCyte® system to examine the dynamics of immune cell killing of cancer cell lines in culture. Using this system, we

cultured nuclear localized red fluorescent-labeled MDA 231 breast cancer cells, obtained from Sartorius, in the presence of IncuCyte® caspase-3/7 reagent (Sartorius, USA) (**Fig. 1A**). The Sartorius Caspase-3/7 reagent, similar to NucView488 [8], is a cell-permeable, non-toxic dye conjugated to the caspase-3/7 recognition and cleavage sequence (DEVD) [12]. It is non-fluorescent until activated by caspase-3/7 apoptosis activity. Once cleaved, the dye is capable of fluorescing green upon DNA binding, thus identifying the dead and dying cells. As a proof of principle that this system works as expected, we treated the cells with a known apoptosis inducing drug, staurosporine (**Fig. S2**). We find that the number of red fluorescent MDA 231 cells increase in number until shortly after the addition of staurosporine whereupon the number of red MDA 231 cells then begin to decrease, and the number of green apoptotic cells increase. These experiments serve as a positive control for the co-culture experiments in which the green cells indicate cell death due to apoptosis. Our data using staurosporine also shows that 1.2 μM staurosporine kills MDA 231 breast cancer cells more effectively than the lower dose of 0.6 μM staurosporine, demonstrating that elevated concentrations of staurosporine are more effective at killing tumor cells *in vitro*.

Using this system, we added activated or non-activated PBMC cells (non-fluorescent) to the Sartorius MDA 231 cells (red fluorescent and treated with caspase-3/7 reagent). Non-activated PBMCs can induce cell death but at much lower level than activated PBMCs generated using OKT3 stimulation (**Fig. 1**).

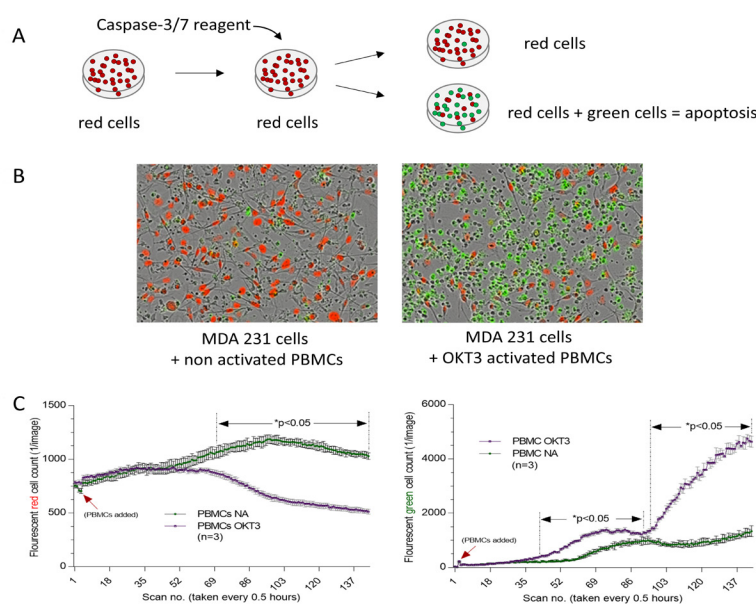


Figure 1. The IncuCyte® live-cell analysis system to study immune cell kill responses of cancer cell lines in real-time. **A.** Schematic representation of the dual color monitoring system to track live cells by fluorescent protein expression (red) and cell death or apoptosis (green) by caspase-3/7 reagent. **B.** Red fluorescent MDA 231 cells (Sartorius) co-cultured with non-activated control or OKT3 activated PBMCs are imaged using the IncuCyte® system. Images are taken 60 h post co-culture with PBMCs or scan No. 120 (see C). **C.** Real-time viability measurements are shown. Non-activated (NA) PBMC control conditions are shown in green circles and OKT3 activated PBMCs shown by red circles. The graph on the left compares the live (red) cells and the graph on the right compares the green (dead) cells between NA and activated PBMCs. 20000 red fluorescent MDA 231 cells and 50000 non-activated control or OKT3 activated PBMCs were cultured. Statistical analysis was performed between NA and activated PBMCs for both cell survival and cell death with *P*-values indicated on the graph. The graph (left) showing live cells has a $P < 0.05$ for every time point from scan 69–140. The graph (right) for cell death has a $P < 0.05$ for time points between scan 40–90 and 100–140. Data is the mean of 3 independent wells per time point \pm SEM. Images were taken at 10 \times magnification on the IncuCyte® imager.

At 60 h post co-culture, there was a significant increase in cancer cell death in the presence of activated PBMCs (Fig. 1B, right panel) compared to a reduction in tumor cell death in the presence of the control non-activated PBMCs (Fig. 1B, left panel). Cell death is indicated by widespread cell clumping of green-fluorescing cells that have cleaved the caspase-3/7 reporter reagent (Fig. 1B, right panel). The rate and degree of cell death was measured in real-time using the IncuCyte® imaging system (Fig. 1C). At approximately 35 h post treatment (scan No. 70), the number of live red cells (Fig. 1C, left graph) began to decrease in the presence of activated PBMCs, whereas the number of live red cells treated with the non-activated PBMC control continued to increase until plateauing at 45 h post treatment. In measuring the green apoptotic cells (Fig. 1C, right graph), the number of green cells significantly increased in the presence of activated PBMCs at 45 h post, whereas the number of green cells treated with non-activated PBMCs remained relatively unchanged. Statistical analysis was performed for both cell survival and cell death measurements between cells co-cultured with non-activated and activated PBMCs with *P*-values indicated on the graphs in Figure 1C. The graph (left) showing live cells has a *P* < 0.05 for every time point from scan 69–140. The graph (right) for cell death has a *P* < 0.05 for time points between scan 40–90 and 100–140. These

results demonstrate the power of the IncuCyte® system in measuring the immune cell-cancer cell killing dynamics.

To test the limits of the co-culture experiments, red fluorescent-labeled MDA 231 were plated at the following MDA/non-activated PBMC cell number ratios: 50000/50000, 50000/20000, 20000/100000, 20000/50000, and 10000/100000 (Fig. S3). As shown, the ratio of 20000/50000 is suitable for testing potential therapeutics since this tumor cell/PBMC ratio does not severely affect tumor cell survival and does not overly induce apoptosis. Therefore, this ratio was used throughout the manuscript.

Generation of stably-labeled red fluorescent cells using a nuclear localized mKate2 expressing lentivirus

To effectively perform tumor cell killing assays with co-cultured immune cells, clear visualization of the cancer cells as well as the effector immune cells is required. Although stably fluorescent cancer cell lines are commercially available for some tumor cell lines, they are expensive and cost prohibitive for many researchers—especially if one desires to study several different cancer cell lines or tumor cells from rare or commercially unavailable tumor biopsies. Thus, we generated our own nuclear localized fluorescently labeled cell lines using lentivirus to stably express the red fluorescent mKate2 protein (Fig. 2).

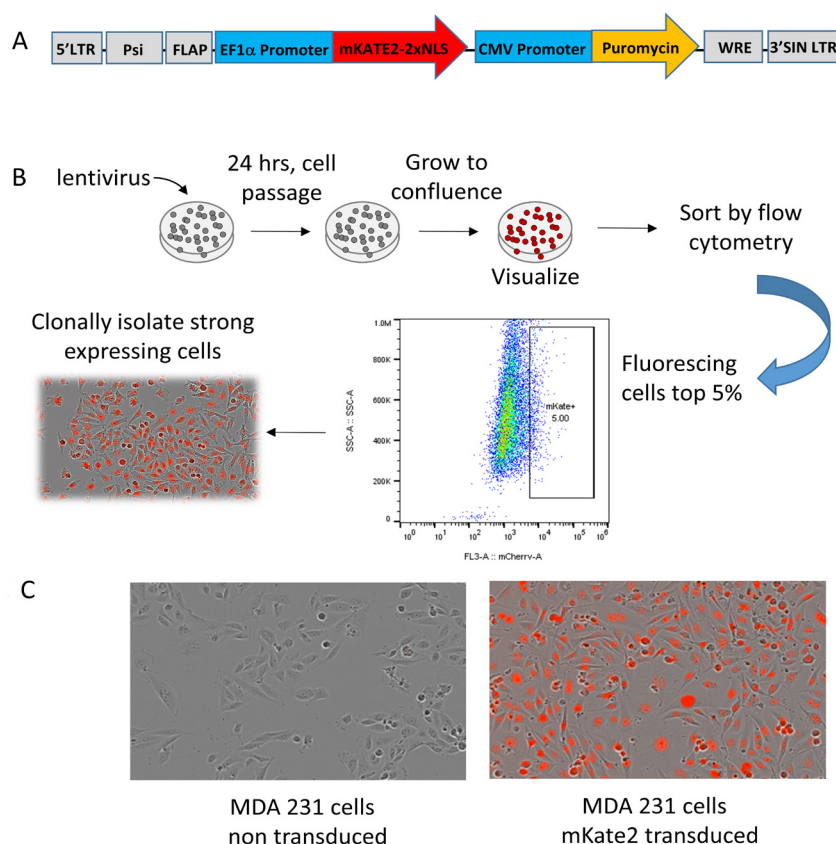


Figure 2. Generation of stably-labeled red fluorescent cells using a nuclear localized mKate2 expressing lentivirus. **A.** Layout of the plentilox EF1 α -mKate 2X NLS-Puro construct. The construct expresses mKate2 fused to two nuclear localization sequences expressed by the EF1 α promoter. A second expression cassette of CMV promoter driving the puromycin selectable marker is included. **B.** Schematic representation of experimental design of generating fluorescently label cancer cell lines. Cells were transduced with lentivirus expressing mKate2, grown to confluence and the strongest expressing cells isolated for expansion. Shown is a FACS diagram sorting the top 5% of fluorescing A375 cells and corresponding image of cells after clonal isolation. **C.** Image of ATCC MDA 231 cells, transduced with lenti-mKate2 and clonally isolated compared to non-transduced control. Images were taken at 10 \times magnification on the IncuCyte® imager.

To generate the lentiviral construct, we inserted the mKate2 fluorescent gene tagged with two C-terminal nuclear localization signals behind the constitutively active EF1 α promoter. The EF1 α promoter was chosen due to its consistently strong expression across a wide range of cell types and resistance to being silenced over time by epigenetic factors such as DNA methylation [14,15]. This construct also contains a CMV promoter driven-puromycin resistance selectable marker cassette (Fig. 2A). The construct was then packaged into lentivirus and used to transduce cancer cells obtained from American Type Culture Collection (ATCC, USA). For cloning, transduced tumor cells were passaged and grown to confluence, then sorted by flow cytometry and clonally plated onto 96 well plates (Fig. 2B). Notably, only the top 5% of cells expressing the highest fluorescence intensity were selected for single cell cloning. Clonal isolates were further monitored for growth using the IncuCyte[®] imaging system. The brightest, best-growing clones were selected for further expansion and analysis. The first cancer cell line generated using this system was MDA 231 cells obtained from ATCC. As shown in Figure 2C, these MDA 231 breast cancer cell lines fluoresce as strongly as the MDA 231 cell line obtained commercially

(see Fig. 1C).

Generation of multiple cancer cell lines transduced with lentivirus mKate2

An effective cell labeling method must consistently work on a diverse set of human cancer cell lines. Multiple lines from ATCC (Table S1) were chosen not only from the same cancer type—but across different cancer cell lines to test for consistency and to test the range of effective cell labeling (Fig. 3). We used lenti-mKate2 to label five breast cancer cell lines: MDA 231 cells (Fig. 2C), MDA 436 cells (Fig. 3A), T47D cells (Fig. 3B), MCF-7 cells (Fig. S4), and BT-474 (Fig. S4); three melanoma lines: A375 cells (Fig. 3C), A375 MA2 cells (Fig. S4), and Mel-1 cells (Fig. S4); one prostate cancer line: (LNCAP cells (Fig. 3D); and two lung cancer lines: A549 cells (Fig. S4) and NCI-H460 cells (Fig. S4). All transfected tumor cell lines were clearly labeled with the red fluorescent mKate2 marker despite different origins, morphologies, and growth rates. Lenti-mKate2 labeled cells were treated with caspase-3/7 reagent and little to no level of background green fluorescence can be detected (Fig. 3).

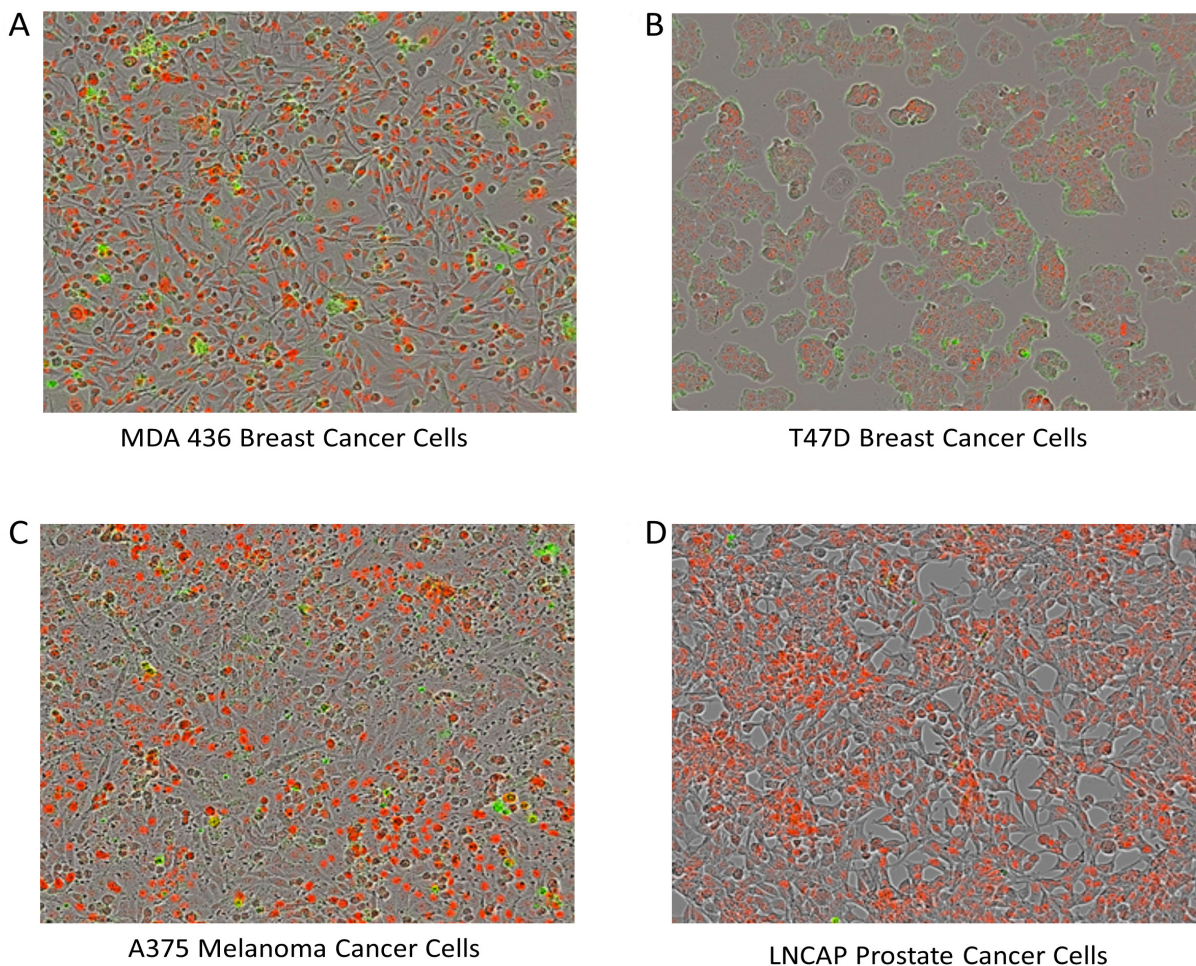


Figure 3. Generation of multiple cancer cell lines transduced with lentivirus mKate2 and clonally isolated. A. MDA 436 breast cancer cells, B. T47D breast cancer cells, C. A375 melanoma cells and D. LNCAP prostate cancer cells were stably labeled with lenti-mKate2. These cells were analyzed on the IncuCyte[®] system for up to 6 d in the presence of caspase-3/7 reagent. Images are taken at the 60 h time-point at 10 \times magnification on the IncuCyte[®] imager.

Functional testing of lenti-mKate2 transduced cancer cell lines in real-time cell viability assays

To functionally assess interactions between lenti-mKate2 labeled cancer cell lines and human immune cells, several cell lines were tested under varying conditions (Fig. 4). First, 20000 MDA 436 breast cancer cells treated with the caspase-3/7 reagent were cultured with (Fig. 4A) or without (Fig. 3A) 50000 non-activated CD3⁺ T cells. Cells were analyzed in real-time by the IncuCyte[®] imaging system and images are

shown at 60 h after treatment with CD3⁺ T cells. There is significantly more cell death in the presence of CD3⁺ T cells as compared to control wells (Fig. 3A, and Fig. 4A, left graph). Concomitantly, there is a modest increase in live cell number of MDA 436 cells with caspase reagent only (Fig. 3A) compared to cells co-cultured with CD3⁺ T cells (Fig. 4A, right graph). Statistical analysis shows a $P < 0.05$ for every time point between scan 20–325 for cell death (left graph) and between scan 70–324 for live cells.

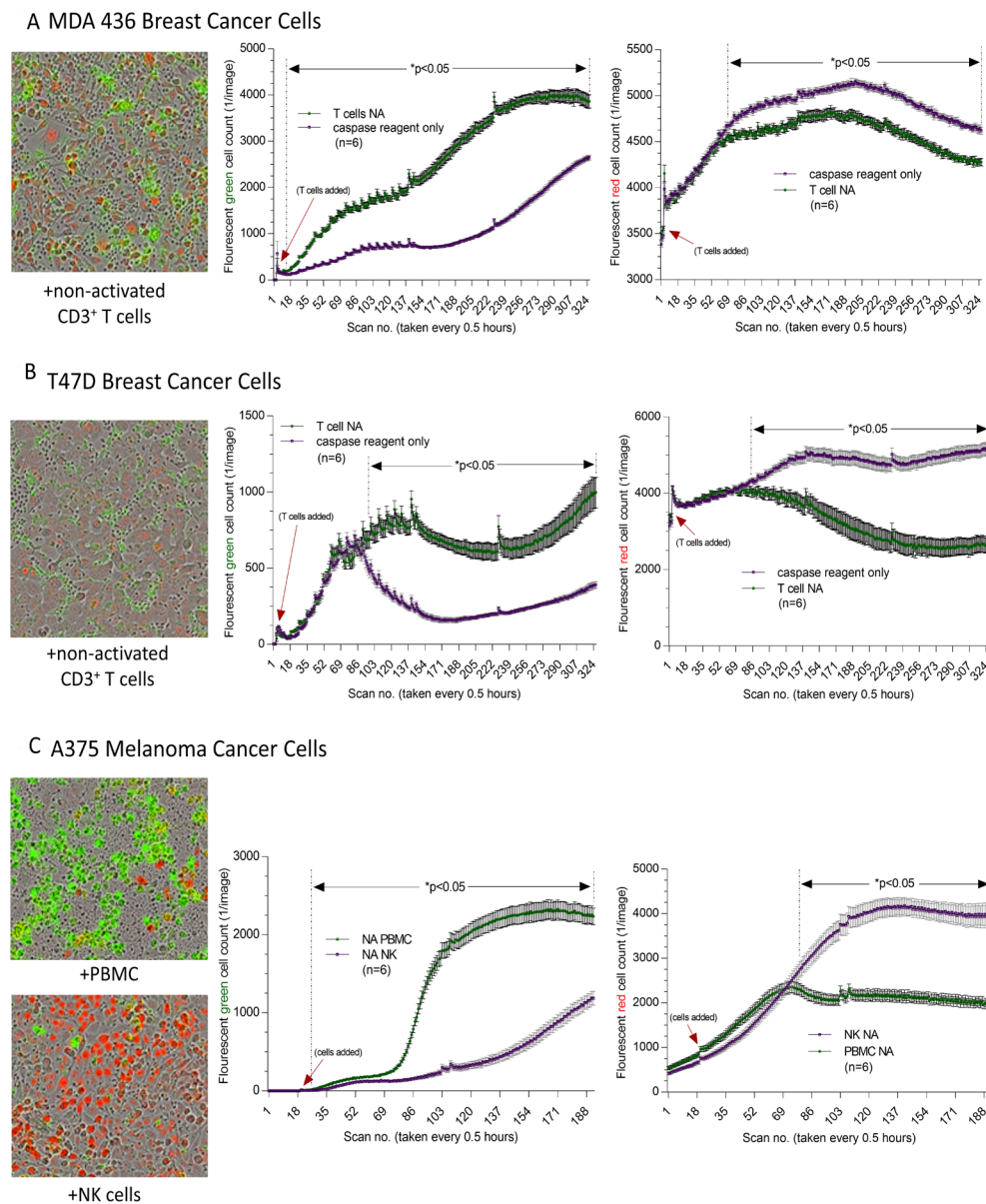


Figure 4. Functional testing of lenti-mKate2 transduced cancer cell lines in real-time cell viability assays. Red fluorescent cancer cell lines are co-cultured with immune cells and analyzed using the IncuCyte[®] system. Breast cancer cells lines MDA 436 (A) and T47D (B) were co-cultured with non-activated CD3⁺ T cells or caspase reagent only as control (compare 4A with 3A and 4B with 3B, respectively). C. A375 melanoma cells were co-cultured with either natural killer (NK) cells or non-activated PBMCs as a positive control. All cells were treated with caspase-3/7 reagent prior to co-culture. Images are taken at the 60 h time point. Real-time viability measurements are shown in the graphs. The graphs on the left compared the dead (green) cells and the graphs on the right compared the red (live) cells between culture conditions. In each experiment, 20000 cancer cells were co-cultured with 50000 T-cells, NK cells or PBMC cells. Results are representative of three biological replicates. Statistical analysis was performed and P -values are indicated on each graph. Each time point (scan) between the arrows indicates a $P < 0.05$. Data is the mean of 3 independent wells per time point \pm SEM. Images were taken at 10 \times magnification on the IncuCyte[®] imager.

A comparable experiment was performed using T47D breast cancer cells resulting in a similar outcome. The growth/death rates were again measured in real-time on the IncuCyte® imager (Fig. 4B). As shown, 20000 T47D cells treated with 50000 CD3⁺ T cells had more cell death and fewer live cells compared to caspase reagent only control wells (compare Fig. 3B to Fig. 4B, Fig. 4B left and right graph, respectively). A $P < 0.05$ was determined for all time points between scan 100–324 for cell death (left graph) and scan 85–324 for live cells (right graph). Finally, we compared the killing potential of 50000 PBMCs and isolated natural killer cells (NK) on 20000 A375 melanoma cancer cells (Fig. 4C). A375 cells were treated with caspase-3/7 reagent and then co-cultured with either non-activated PBMCs as a positive control or NK cells. Tumor cells were monitored in real-time on an IncuCyte® imaging system for 94 h. Analysis of the green cells shows a significant increase in cell death when co-cultured with PBMCs, as expected, but not significant cell death with NK cells (Fig. 4C, left graph) ($P < 0.05$ from scan 25–188) in which A375 cells (red) continued to grow in the presence of NK cells. By contrast, A375 cell numbers plateaued sooner in the presence of the PBMC positive control than with NK cells (Fig. 4C, right graph) ($P < 0.05$ from scan 75–188). Images of A375 cells at 60 h post co-culture shows the majority of cells have turned green when treated with PBMCs, but remain mostly red when treated with NK cells. Overall, these experiments demonstrate that interactions between the lenti-mKate2 labeled cells and immune cells can be readily visualized and quantitated in real-time.

DISCUSSION

Live-cell imaging analysis allows for the study of cellular dynamics in real time. These powerful techniques depend on live tracking of target and effector cells. Parameters such as cell growth, death, migration and intracellular and extracellular trafficking can be measured in real time instead of static snapshots of single time points. The standard approach is to use stably transfected fluorescently labeled cells, usually with high intensity. This relatively simple component can be the limiting factor to utilizing the full potential of live-cell imaging of fluorescently labeled cancer cells. Although, these cells are commercially available, purchasing such cells can be very expensive and therefore cost prohibitive, especially for investigations that are designed to test a single therapeutic on a wide variety of tumor cells. Secondly, the number and type of commercially available cancer cell lines appropriately labeled for use in a cell imaging system are very limited. Thirdly, available cancer cell lines may have different and/or unwanted genetic backgrounds (*e.g.*, p53 mutation vs. Ras mutation) [16,17] that may or may not be relevant, or unnecessarily interfere with experimental interpretation and/or results. These factors were paramount when designing the pLentilox EF1 α -mKate 2X NLS-Puro construct. The mKate2 fluorescent marker was specifically used for its high intensity fluorescence required for quality live-cell imaging. The nuclear localization of mKate2 is essential for distinct cells to be clearly delineated for superior masking of cells in the live-imaging experiments. Cell masking is process where the imager is programmed to measure just the cells of interest, blocking out all other cells and debris. This process is essential for obtaining accurate data. In addition, it is common for stably derived cell lines to epigenetically silence expression over time by DNA methylation of the promoter. To minimize the chance of this occurring, we used the housekeeping gene promoter, EF1 α , which is more resistant to silencing than strong consti-

tutively active viral promoters, which are commonly used in lentiviral constructs. These characteristics of the pLentilox EF1 α -mKate 2X NLS-Puro create fluorescently-labeled cells that are superior to other lentiviral-generated fluorescently-labeled cells for live-cell imaging that we examined in the IncuCyte®.

To maximize the benefits of the pLentilox EF1 α -mKate 2X NLS-Puro construct, our methods were designed to isolate the highest expressing clones that optimize the cellular fluorescence of the resultant tumor cells. Our transduced cells are comparable in fluorescence to those obtained commercially, and functioned similarly when tested in co-culture using an IncuCyte® cell imaging system. The ten cell lines we transduced with lenti-mKate2 were clonally isolated fairly easily using standard cell culturing techniques and reagents. For tumor cells that cannot grow from a single cell clone, this method should work by cell sorting the top 5% of fluorescent cells and growing them as a heterogeneous population. From these studies, we were able to show that this method is a high quality, high efficiency and low cost approach for generating fluorescent cells from multiple types of cancers.

Recent studies investigating the interactions of cytotoxic T lymphocytes with melanoma tumor cells [18] and a cell-engineered system to assess tumor cell sensitivity to CD8⁺ T cell-mediated cytotoxicity [19] were done using the standard methodology. Neubert *et al.* used flow cytometry to measure living cells and chromium release assay to evaluate cell killing. These data were limited by the number of time points (3 or 4) for each experiment. The error bars for some points are substantial, although not unusual for these types of studies. Furthermore, the chromium release assay used radioactive chromium (⁵¹Cr) in the experiment. Similar experimental limitations were observed in Nelson *et al.*, which used FITC annexin V for apoptosis detection to measure apoptotic cell death. These older methods may be limited by the number of time points, the reproducibility of results, and in some cases, the dependence on radioactivity. Use of live-cell imaging reduces or eliminates these disadvantages. Hundreds of time points can be measured, across several replicates with great reproducibility as indicated by the small error bars at each time point. Data is captured and processed by the IncuCyte®, eliminating time consuming assays, and radioactivity is not required.

The search for immuno-therapies against cancer is an active area of research [2,3]. In order to maximize the effectiveness of these targeting strategies, a robust system for labeling cancer cells in a rapid and cost effective manner is essential. Live-cell imaging technologies that can visualize, characterize and quantitate interactions between immune cells and tumor cells may identify the most effective therapeutic regimens for treating cancer. These fluorescent labeling systems can also be used to evaluate the efficacy of antibody based therapies for cancers that do not respond to traditional treatments. They can also contribute as a valuable drug development tool for identifying and improving our understanding of the binding interactions between lymphocytes and tumor cells (*i.e.*, receptor-ligand interactions). Moreover, the potential of these labeling systems can also lend themselves to testing other relevant therapies apart from immune based therapeutic approaches for cancer.

Lastly, translational applications for customized medicine using these approaches may help identify when resistance occurs in patients—and aid in the development of novel, multi-tiered approaches for treatment of several cancers. This system can also be used to understand how therapies affect neighboring non-cancer cells in comparison to cancer cells in real time. A two color assay system for concurrently measuring

living cells, and cell death, can provide a greater degree of confidence in the cellular dynamics under different co-culture conditions. As live-cell technologies advance, the addition of multiple fluorescent reporters would add depth to understanding concomitant processes in the search for more effective cancer therapies, as well as advance our understanding of critical biological pathways in tumor cells.

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Supplementary information

Figure S1. Construct map, sequence and primer design for pLentiLox EF1 α -mKate 2X NLS-CMV-Puro.

Figure S2. MDA 231 breast cancer cells treated with apoptosis inducing staurosporine.

Figure S3. Multiple plating densities for MDA 231 breast cancer cells in co-culture with PBMCs.

Figure S4. Additional cell lines transduced with lentivirus mKate2 representing melanoma, breast and lung cancers.

Table S1. Cell lines used to generate red fluorescent lines using lentivirus-mKate2.

Supplementary information of this article can be found online at <http://www.jbmethods.org/jbm/rt/suppFiles/323>.



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