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Genetic Barcoding with Fluorescent Proteins for Multiplexed Applications

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Abstract:	Fluorescent proteins, fluorescent dies and fluorophores in general have revolutionized the field of molecular cell biology. In particular, the discovery of fluorescent proteins and their genes have enabled the engineering of protein fusions for localization, the analysis of transcriptional activation and translation of proteins of interest, or the general tracking of individual cells and cell populations. The use of fluorescent protein genes in combination with retroviral technology has further allowed the expression of these proteins in mammalian cells in a stable and reliable manner. Here we show how one can utilize these genes to give cells within a population of cells their own biosignature. As the biosignature is achieved with retroviral technology, cells are barcoded ´indefinitely´. As such, they can be individually tracked within a mixture of barcoded cells and utilized in more complex biological applications. The tracking of distinct populations in a mixture of cells is ideal for multiplexed applications such as discovery of drugs against a multitude of targets or the activation profile of different promoters. We show how to elegantly develop and amplify barcoded mammalian cells with distinct genetic fluorescent markers, how we can use several markers at once or one marker at different intensities and finally, how the cells can be further utilized in combination with cell-based assays to increase the power of analysis through multiplexing.
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Cover Letter

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Submission of manuscript by Smurthwaite, CA *et al* to the Journal of Visualized Experiments

To the Editor/s of Jove:

I am pleased to submit our manuscript entitled 'Genetic Barcoding with Fluorescent Proteins for Multiplexed Applications' by Cameron Smurthwaite, Wesley Williams, Alexandra Fetsko, Darin Abbadessa, Zachary Stolp and Roland Wolkowicz to the Journal of Visualized Experiments. In this manuscript we describe in detail how we have obtained the data described in our recently published manuscript in the journal of Cytometry part A. Here we further describe in a more visualized and descriptive manner how we utilized our fluorescently genetically barcoded cells to produce panels of distinct populations, and the importance of selection of fluorescent proteins and correct compensation. We also show in a new experiment, how a panel of fluorescently barcoded cells is used for an independent assay in a multiplexed format. We consider this article to have a strong impact in a visualized fashion. We thus believe this manuscript is of interest to the scientific community and we are delighted to submit it for your consideration in JoVE.

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Wesley Williams, Discussed results and wrote manuscript
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TITLE

Genetic Barcoding with Fluorescent Proteins for Multiplexed Applications

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genetic barcoding; fluorescent proteins; retroviral technology; high-throughput; flow cytometry; multiplexing

SHORT ABSTRACT:

Since the discovery of the green fluorescent protein gene, fluorescent proteins have impacted molecular cell biology. This protocol describes how expression of distinct fluorescent proteins through genetic engineering is used for barcoding individual cells. The procedure enables tracking distinct populations in a cell mixture, which is ideal for multiplexed applications.

LONG ABSTRACT:

Fluorescent proteins, fluorescent dyes and fluorophores in general have revolutionized the field of molecular cell biology. In particular, the discovery of fluorescent proteins and their genes have enabled the engineering of protein fusions for localization, the analysis of transcriptional activation and translation of proteins of interest, or the general tracking of individual cells and cell populations. The use of fluorescent protein genes in combination with retroviral technology has further allowed the expression of these proteins in mammalian cells in a stable and reliable manner. Shown here is how one can utilize these genes to give cells within a population of cells their own biosignature. As the biosignature is achieved with retroviral technology, cells are barcoded ´indefinitely´. As such, they can be individually tracked within a mixture of barcoded cells and utilized in more complex biological applications. The tracking of distinct populations in a mixture of cells is ideal for multiplexed applications such as discovery of drugs against a multitude of targets or the activation profile of different promoters. The protocol describes how to elegantly develop and amplify barcoded mammalian cells with distinct genetic fluorescent markers, and how to use several markers at once or one marker at different intensities. Finally, the protocol describes how the cells can be further utilized in combination with cell-based assays to increase the power of analysis through multiplexing.

INTRODUCTION:

Technologies such as fluorescence spectroscopy, fluorescence microscopy and flow cytometry, all rely on fluorescence, a property widely exploited in biochemical, biomedical, and chemical applications. Fluorescence, whether intrinsic or through labeling, has been exploited for the analysis of protein expression profiles and patterns, cell fate, protein interactions and biological functions [1-9], and, through fluorescence/Förster resonance energy transfer, for the detection of biomolecule interactions and conformational changes[10-13]. Since the isolation of the *Aequorea victoria* green fluorescent protein (GFP)[14], the discovery of additional naturally occurring fluorescent proteins from other cnidarians, particularly corals, has largely increased the number of existing fluorescent proteins with distinguishable excitation/emission spectra. These, together with the introduction of mutations in their genes[15-19], have further

expanded the possibilities, obtaining a true palette of fluorescent proteins available to scientists that exploit microscopy, flow cytometry and other fluorescence-based technologies for their research.

In parallel, although independently, the development of retroviral technology has drastically facilitated the stable expression of ectopic genetic information in mammalian cells[20-23]. It is thus not surprising that this technology has been used to transfer genes of fluorescent proteins into a broad number of cell types and tissues[24-28] or for production of transgenic animals[29-31]. Following the nature of retroviruses, the genetic information of the ectopic fluorescent protein is introduced within the genome of the cell[32] and the cell becomes fluorescent `for ever´. This property has allowed tracking of cell fate, or of a single cell within a population of cells. The now fluorescent cell has thus acquired its own biosignature and can be defined as barcoded. Its unique biosignature identifies it from other cells, and importantly, distinguishes it from cells genetically manipulated to express different fluorescent proteins with distinguishable absorption/emission spectra. Biological applications such as the tracking of reprogramming factors toward pluripotency[33], the analysis of subnuclear factors for the elucidation of nucleolar localization[34], the construction of fluorescent reporter plasmids for transcriptional studies[35] or the genetic labeling of neurons for the study of neuronal network architecture[36], are just four examples of the many that have exploited different fluorescent protein genes for the same experimental setup.

Flow cytometry has been broadly utilized for the analysis of biological processes at the single cell level, such as gene expression, cell cycle, apoptosis, and signaling through phosphorylation[37-43]. The stable expression of fluorescent protein genes in mammalian cells has further enhanced the utility of flow cytometry for cell analysis[38, 44] and ligand-receptor interactions[45]. Enhanced capabilities have allowed flow cytometry to become a widely utilized methodology for high-throughput and high-content screening[46]. But despite the now expanded number of fluorometers and robotics technologies that can couple plate reader systems, imaging and flow cytometry, there seems to be a lack in experimental design that can exploit and fit these enhanced technological capabilities.

Fast, reliable, simple and robust cell-based methodologies are drastically needed for multiplexed applications that further enhance high-throughput capacity. This is especially true in the field of drug discovery where engineering cell-based assays in a multiplexed format can enhance the power of high throughput screening[39, 47-50]. Multiplexing, as it allows simultaneous analyses in one sample, further enhances high-throughput capabilities[51-54]. Fluorescent genetic barcoding not only allows for elegant multiplexing, but also, once engineered, circumvents the need of time-consuming protocols, reduces costs accompanied with antibodies, beads and stains[39, 52, 55], and can reduce the number of screens required during high throughput screening. We have recently described how retroviral technology can enhance multiplexing through fluorescent genetic barcoding for biological applications, by expressing an assay previously developed to monitor HIV-1 protease activity[56, 57] with different clinically prevalent variants[58]. The methodology is explained in a more descriptive manner focusing on how to select and amplify genetically fluorescent barcoded cells and how to

produce panels of clonal populations expressing distinct fluorescent proteins and/or different fluorescence intensities. Panels of cell populations distinguishable based on their fluorescent characteristics enhance multiplexed capabilities, which can be further exploited in combination with cell-based assays that tackle different biological questions. The protocol also describes how to engineer a panel of barcoded cells bearing one of the cell-based assays previously developed in the laboratory, as example[59]. This protocol is thus not intended to show the well-established retroviral/lentiviral technology for genetic transfer, the value of fluorescent proteins or the applications of flow cytometry[60, 48] but rather to show the enhancing power of combining the three for multiplexed applications.

PROTOCOL:

1. Preparation of Mammalian Cells, Viral Production and Transduction for Genetic Barcoding

1.1) Plate $2.5x10^6$ adherent cells in a 10 cm plate (or around 50 to 60% confluency) one day prior to transfection in Dulbecco's modified eagle medium (DMEM) with 10% fetal calf serum (FCS). For retroviral production use packaging cell-line of choice such as Phoenix-GP (a kind gift from Gary Nolan, Stanford University).

Note: The packaging cell line stably expresses Gag and Pol proteins for retroviral particle formation *in trans*. Make sure cells are healthy and adhered to the plate in a monolayer, prior to transfection.

1.2) 24 hrs later, prepare the DNA transfection mixture.

1.2.1) To 125 µL of serum-free DMEM add 3 µg Vesicular Stomatitis Virus Envelope glycoprotein vector (pCI-VSVg) and 3 µg of transfer vector carrying the fluorescent protein gene of interest. Mix and add 15 µL of polyethylimine (2 mg/mL).

1.2.2) Incubate mixture at room temperature for 15 min and add to cells drop-wise.

Note: Polyethylimine is added to the homogenous DNA mixture as transfection reagent for the formation of polyplexes. In order to obtain viral particles carrying different fluorescent protein markers, perform each transfection independently with the marker of choice. Remember that the retroviral transfer vector is around 7 Kb in length and cannot be packaged if above 10 Kb.

1.3) Incubate plates at 37 °C. In order to increase viral production place plates at 30 °C or 32 °C instead.

Note: 24 hours post transfection media can be replaced with 7 mL of fresh media, and although this may improve cell health it may decrease viral load in the supernatant.

1.4) 48 hrs post transfection collect the supernatant-containing viral particles and filter with a 0.45 µM polytetrafluoroethylene filter. Use freshly collected viral supernatant for transduction. Otherwise keep supernatant in aliquots at -80 °C and avoid freeze-thaw. Remember that viral titer will decrease probably up to 50% with each freezing-thawing cycle.

1.5) Plate cell line of choice 24 hrs prior to transduction if adherent, or immediately before transduction if non-adherent. Seed 2.0 $x10^5$ to 3.0$x10^5$ adherent cells (here Huh 7.5.1 and HEK 293T) or 5.0$x10^5$ to 1$x10^6$ non-adherent cells (here SupT1) per well in a 6-well plate.

Note: Make sure to calibrate the number of cells to be seeded prior to transduction, especially so for adherent cells, so cells achieve a confluency of around 60% at the time of transduction.

1.6) Add 5 µg/mL Polybrene (hexadimethrene bromide) to seeded cells and then add previously collected viral supernatant to obtain around 20% to 40% infection (here 2 mL).

Note: The MOI or percentage of infection is mostly arbitrary, as sorting will allow to amplify any subpopulation of cells. Obviously, a higher rate of infection eases and speeds up the process of amplification.

1.7) Transduce cells by centrifugation in a hanging bucket rotor at 1,500 x g for 120 min at 32 °C. Resuspend non-adherent cells with fresh media prior to incubation. For adherent cells, replace with fresh media 24 hr post-transduction.

Note: It is recommended to seal the plates prior to centrifugation with parafilm to avoid spilling over. To increase genetically barcoded cell diversity, transduce cells with viral particles carrying different fluorescent proteins (obtained from step 1.4). When choosing fluorescent markers, make sure they are compatible with the instrumentation and they have distinguishable fluorescent parameters.

2. Selection and Amplification of Genetically Barcoded Cells

2.1) Analyze transduced mammalian cells by flow cytometry at least 72 hrs post transduction to quantitate the percentage rate of transduction. Use a comparable non-transduced cell line as negative control.

Note: As sorting will be used to purify individual barcoded cell populations, a high percentage rate of initial transduction, while not necessary, should speed up the process of amplification.

2.2) Utilize the cell-sorter of choice to obtain the cells that express the protein of interest.

2.2.1) For that purpose, ensure the gates are properly set in the right channels.

2.2.1.1) To do so use the same naïve cell line as negative control and set the parameter/channel voltage so that the negative population is below the value of 10^3 on each fluorescent axis. Determine gating for positive fluorescence as events that appear above that of the negative control for each fluorescent channel.

2.2.2) Be aware that each instrument may vary and the correct voltage for determining gating should be adjusted accordingly, making positive and negative controls imperative in every experimental setup.

2.2.2.1) For fluorescence detection in this experimental setup use the FITC channel (530/30 band pass filter proceeded by a 502 long pass filter) for eGFP, the PE channel (585/42 band pass filter proceeded by a 556 long pass filter) for td Tomato, and the APC channel (660/20 band pass filter) for E2 Crimson.

Note: The 488nm laser excites eGFP and td Tomato while E2 Crimson is excited by the 633nm laser.

2.3) Sort into 15 mL conical tubes with 1mL of 100% FCS.

Note: The process of sorting fluorescent cell populations is not compulsory as one can directly proceed with the sort of individual clones (see below). Sorting tight populations based on chosen fluorescence ensures a backup population for future selection of individual clones. Keep in mind that individual cells are at times difficult to grow while a large population of cells can be easily frozen, and then thawed and amplified at a later stage.

2.4) Spin down the sorted populations in a hanging bucket rotor centrifuge at 524 g at 32 °C for 5 minutes to pellet cells. Re-suspend in 1 mL of fresh media, and re-plate cells so that cell confluency is below 60% to allow growth and division for at least two days.

2.4.1) For example, plate adherent cells at around $3x10^5$ cells in 2 mL of media per well in a 6-well plate and $2x10^6$ cells in 2 mL of media in a 6 cm plate. Use DMEM for adherent cells and RPMI for non-adherent cells (or any specified medium if required). Place plated cells in an incubator at 37 °C for several days to allow amplification.

3. Obtain Clonal Populations of Genetically Barcoded Cells at Different Intensities

3.1) Obtain clonal populations from cells originally transduced (step 1.7) or from the sorted population (step 2.2).

Note: This will ensure equal or similar level of fluorescent protein expression among all the cells within the population (a tight population with a small CV in mean fluorescence intensity of up to 40%).

3.1.1) For that purpose, sort single cells into 96-well plates with an automated cell deposition unit (ACDU). Set gates for sorting according to populations of interest. Ensure gates are set at least one log-scale apart to obtain distinguishable populations when choosing cells at different fluorescent intensities. For the procedure shown here, use the same flow cytometry experimental set up as the one described in step 2.2.

3.2) Amplify individual clones in an incubator at 37 °C for at least two weeks. Through the process of amplification analyze the cells under the microscope to ensure that growing populations arise from individual cells and not from more than one.

Note: Remember that the sorter will place one cell per well on average, and while some wells may not have any cell at all, others may have more than one.

3.2.1) To ensure that a population arises from one cell only, be extremely gentle with the plate and never disturb the wells by pipetting. Observe the plate, well by well, under the microscope.

Note: While individual cells may at times be difficult to identify, once they start dividing they will be easily recognizable.

3.2.2) Be aware that often cells 'clump' along the edges. Discard those wells where multiple clonal populations can be observed and keep those that have one tight population.

Note: It is advisable to transfer those into larger plates for amplification.

3.3) Screen the putative clonal populations by analyzing them by flow cytometry. Compare them to a negative control utilizing the same flow cytometry experimental setup.

3.3.1) Analyze only a percentage of the sample and keep the rest as backup. Choose the most distinctly separate populations based on average intensity and tightness of the population. Amplify them as needed or freeze stocks in freezing media with 20% glycerol at -80 °C for short periods of time or liquid nitrogen for longer.

Note: Remember that a 'true' clonal population should have a very tight fluorescence pattern.

4. Ensure Multiplexing Capabilities for High Throughput Screening (HTS)

4.1) Combine as many distinct clonal populations as needed that can be further utilized in a multiplexed format. Choose clones with distinguishable absorption/emission spectra and/or different intensities. If a 96-well plate format is used, which is suitable for HTS, seed 50,000 cells in 200 µL per well.

Note: Keep in mind that the number of cells is only an estimate and may change based

on cell-type and whether it is adherent or not.

4.2) Analyze the sample using flow cytometry to ensure that each of the independent established fluorescent cell lines can be distinguished from each other. Prior to the analysis prepare negative and single color controls to set parameters and compensation values.

Note: Setting the parameters to clearly distinguish the mixed populations will enable them to be further utilized in a multiplexed format.

5. Adapt Genetically Barcoded Cell Lines to the Biological Application of Choice

5.1) Transduce established genetically barcoded cell lines with viral particles that contain the assay elements of choice, as described here in Stolp *et al,* 2013[59]. Remember that the assay of choice should occupy an additional and distinguishable fluorescent channel and thus it is to be transferred to the appropriate barcoded cell line.

Note: Each barcoded cell line can bear a different assay.

5.2) Sort genetically fluorescent barcoded cells for those that contain assay elements.

Note: Assay elements can be engineered coupled to a tag or fluorescent protein (as a fusion or through an internal ribosome entry site) for straightforward detection through western blotting, flow cytometry or microscopy. The system utilized in this protocol relies on HA epitope surface expression alone, or with additional FLAG epitope expression.

5.2.1) Stain the clonal populations containing the assay with HA and APC-fluorescently coupled antibodies. Then analyze the clonal populations with anti-FLAG and FITC-conjugated antibody staining.

5.2.2) To track back the assay, analyze or 'decode' the populations in the appropriate channel where the distinct genetically barcoded cell lines can be distinguished. Here, decode the nature of the substrate by analyzing in the PE channel for td Tomato detection.

REPRESENTATIVE RESULTS:

Multiplexing fluorescent genetically barcoded cells for the purpose of biological applications can only be achieved once individual clonal populations have been generated. Multiplexing is most effective when barcoded populations have clear distinct fluorescent characteristics with minimal spectral overlap. The example shown in **Figure 1** with clonal populations of mammalian SupT1 cells illustrates that barcoded cells with mCherry and cyano fluorescent protein (CFP) can be easily analyzed simultaneously without losing their individual fluorescent characteristics. This matrix thus exemplifies a panel of fluorescent genetically barcoded cells that is usable for multiplexing biological applications with a readout in yet an additional available channel. In order to obtain such

a panel it is important to remember the nature of retroviral technology, which will lead to variable ranges of fluorescent intensities within the population due to insertional effects and/or MOI. **Figure 2** illustrates that initial transduction of mammalian cells with viral particles containing either td Tomato or E2 Crimson results in cells that express either one or both of the fluorescent proteins at a wide range of intensities (left panel). Selection and sorting of single cells into 96-well plates as shown by the gated boxes (mid panel), allows one to obtain tight clonal populations following expansion (right panel). A tight population should be defined as having a small CV which may range according to cell type, normally 30 to 40% in mean fluorescence intensity. **Figure 2** also illustrates that to further increase the number of genetically barcoded populations with a matrix of only two fluorescent proteins, one can also exploit fluorescence intensity. Generally, one log deviation in the mean fluorescence intensity between populations is suitable for achieving appropriate separation from each other following sorting and amplification. Td Tomato was chosen in the shown example to illustrate this feature, where two populations of differing intensities, mid and high, were obtained for multiplexing with E2 Crimson at a single intensity.

Multiplexing is a powerful tool to facilitate the analysis of many samples at the same time and for the ability to decode masked populations. Enhancing multiplexing can be achieved with yet a third fluorescent protein such as eGFP, as long as spectral properties do not interfere with each other. In the experimental procedure illustrated in **Figure 3** the panel of six populations obtained with td Tomato and E2 Crimson was exploited to increase multiplexing with eGFP. Retroviral technology was used to transfer eGFP to the populations represented in one of the matrices. When observed in the eGFP channel, the non-green naïve six-population matrix (upper left panel in **Figure 3**) is indistinguishable from the eGFP-transduced population matrix (lower left panel in **Figure 3**). Importantly, the panels can be analyzed in the channels occupied by the original genetic barcode (td Tomato and E2 Crimson). While indistinguishable in these channels (compare populations 1-6 with populations 7-12 in mid panels, **Figure 3**), the individual populations can be analyzed in the eGFP channel, and decoded or tracked back, as shown in the histograms (right panels in **Figure 3**). After repeating the process of transduction, selection, sorting, and amplification, taking advantage of the unoccupied channels is useless if right compensation is not applied to adjust for possible spectral overlap. To prove this, when populations 1, 2, 3 (non-green) and 7, 8, 9 (green) are analyzed in the eGFP and td Tomato channels, populations 7 and 8 are difficult to distinguish (left panel in **Figure 4**). It is thus necessary to choose naïve cells (population 1) as a negative control so that the parameters of the instrumentation can be set. When analyzing any matrix, especially with fluorophores that spectrally overlap, it is imperative that single color controls are used to determine the correct compensation values. In the analysis of **Figure 4** populations 2 or 3, and 7 serve this purpose, allowing to better define populations that are truly double positive(populations 8 and 9).

Genetically barcoded cells with fluorescent markers retain their own identity as defined by their biosignature, when analyzed in the right fluorescent channel with the right instrument. However, the biosignature becomes just an additional individual property unless exploited for biological applications. In order to prove the power of fluorescent

genetic barcoding for multiplexing we decided to introduce one of our previously developed assays for drug discovery into some of the fluorescent barcoded mammalian cell lines. By doing so, fluorescent genetic barcoding was exploited to achieve three assays in one sample. In this procedure a scaffold protein containing one of three putative viral substrate for proteolysis was transferred into the genetically barcoded cells. In the assay, cleavage is revealed by the loss of the FLAG epitope on the cell surface (**Figure 5B**). In contrast, FLAG surface stain represents lack of cleavage. **Figure 5** illustrates the result of the analysis of three different substrates; HIV-1 Envelope wild type (Env wt), HIV-1 Envelope mutant (Env mut), and Dengue Virus (Denv) pr-M. Each of them was introduced into one of three barcoded cell lines, a naïve one and two additional ones utilizing td Tomato at different intensities (mid and high) (**Figure 5A**). Staining for FLAG surface expression reveals which of the substrates was cleaved based on the respective barcode. In the example only HIV Env mut retains the FLAG tag (positive following staining), as seen in the green-FITC channel (**Figure 5C**, right bottom panel). The analysis of the assay can thus be performed independently of the original barcoding, and be further exploited to decode and track back the distinct populations. This method of multiplexing thus relies on an additional channel reserved for the biological readout of interest.

Figure Legends:

Figure 1. Barcoding for multiplexing. Individual populations genetically barcoded using distinct fluorescent proteins such as mCherry, CFP or both (left panel) can be mixed, analyzed, and decoded (right panel) in their respective channels via flow cytometry. Adapted from Smurthwaite, C. *et al.* 2014[58].

Figure 2. Sorting and amplification of barcoded cells. Mammalian cells were analyzed 48 hrs following transduction with viral particles containing either td Tomato or E2-Crimson (left panel). Gates are then set for sorting to include cells expressing td Tomato at different intensities, with/out E2-Crimson (middle panel). Clones from the sorts were amplified and re-analyzed to generate a matrix of six distinguishable populations (right panel).

Figure 3. Decoding reveals masked populations. The matrix of six populations obtained in Figure 2 (td Tomato and/or E2-Crimson) can be further engineered to express an additional fluorescent protein such as eGFP. **A.** The original matrix, when analyzed in the eGFP channel (left panel) is negative and the six populations are indistinguishable from each other. Each of the six populations can be independently analyzed in the eGFP channel as shown in the histograms (right panels). **B.** The same matrix, now expressing also eGFP, was analyzed as in **A**, revealing now their green fluorescent characteristic. Adapted from Smurthwaite, C. *et al.* 2014[58] .

Figure 4. Compensation ensures correct separation. Some of the populations originally chosen based on td Tomato and/or eGFP expression (populations 7 and 8) cannot be properly separated when analyzed together (left panel) unless appropriate compensation for these channels is adjusted (right panel).

Figure 5. Selection of barcoded cells for further adaptation to chosen assay. A. Selection and adaptation to assay of choice. Populations genetically barcoded with td Tomato at different intensities (top left panel) were used for biological applications. Each of the populations was further engineered to contain an assay that monitors cleavage, but each one of a different substrate (top right panel). **B.** Depiction of the assay. Positive FLAG stain indicates lack of cleavage while negative FLAG stain indicates cleavage. **C.** Analysis of the assay following FLAG staining. Mixed populations are distinguishable based on the td Tomato barcode but indistinguishable in the FITC channel (left panels). When stained with FLAG-FITC antibody, only one population is positive for FLAG. Decoding reveals, based on genetic barcoding, that this population bears the HIV Env mut substrate (right panel).

DISCUSSION:

Here two well-established procedures have been combined; genetic engineering through retroviral technology and fluorescent proteins. Fluorescent protein-based genetic barcoding for the production of unique cell lines provides a robust and simple way for multiplexed applications. Generating genetically engineered barcoded cells through retroviral technology, is initially a lengthy process, but allows one to obtain, once established, a reliable and stable source of cell material. The nature of this technology consistently produces genetically engineered cell lines with steady fluorescent characteristics that become their own true distinguishable biosignature.

Both adherent and non-adherent cell types can be barcoded utilizing fluorescent proteins that are easily distinguishable based on their physical absorbance/emission spectra. These include, but are not restricted to proteins such as CFP, mCherry, E2 Crimson and td Tomato. Once genetically barcoded with their distinguishable fluorescent protein, they can be combined to produce a panel of cell populations. Importantly, the panel contains cell populations with the exact genotypic make-up but differentiated only by their fluorescence trait. As such, these panels are perfectly suited for multiplexed applications, greatly enhancing high throughput capabilities.

Due to the different insertional preferences of the retroviral particle into the host genome, coupled with differences in MOI, variable fluorescence intensities from the carried particular fluorescent protein gene are expected. Analysis of a genetically engineered population carrying a unique fluorescent gene will thus detect a range of expression profiles that can be defined based on fluorescence intensity. This differential expression profile can be exploited to create populations that are spectrally distinct from one another. One can thus combine choice of fluorescent markers with fluorescence intensity. Here, matrices of four, six, and twelve-populations, which include two six-population panels of E2 Crimson and td Tomato, with or without eGFP, are shown. In theory, this can be further expanded with different intensities of eGFP as well to create populations of three different fluorescent proteins; each one with different intensities associated with it. As stated previously, consideration should be taken when choosing the fluorescent proteins in order to ensure that separation can indeed be achieved.

Chosen proteins should have distinct absorbance and emission spectra; however, when two spectrally distinguishable but similar fluorescent proteins are used, compensation should be applied. Importantly, proper compensation, which allows for physical separation of emission/absorbance spectra among different fluorophores or fluorescent proteins, is accomplished with the use of appropriate single color controls for detection via flow cytometry.

Multiplexing with fewer fluorescent proteins but exploiting a variety of intensities frees additional channels that can be then further exploited for the biological application of interest. This can increase flexibility of the experimental set-up and simplify the choice of the combination of fluorescent markers to be used. For example, the multiplexed assay shown in **Figure 5** relies on antibody staining, in this case coupled to FITC. As only the PE channel is occupied by genetic barcoding, one can utilize FITC-conjugated antibodies, or conversely, APC-coupled, a decision that can be made based on the appropriate instrumentation and/or antibody availability.

While the technological achievements in the field of flow cytometry, microscopy or combined methodologies are impressive, the bottleneck seems to be the available cell technology for biological applications and HTS. Retroviral technology coupled with the expanding number of fluorescent proteins can be merged to answer this query. Fluorescent genetic barcoding drastically facilitates multiplexing, which, in turn, satisfies the increasing need for expanding HTS capabilities of cell-based assays. Genetic barcoding leads to a robust, cost effective, and simple way to couple cell-based assays with high throughput methodologies for biological applications and drug screening. The power of performing one rather than three screens using a three-population panel has beneficial cost and time-related implications. With the growing versatility in flow cytometry, high-content imaging and flow-cytometry-coupled microscopy, fluorescent genetic barcoding will be a consistently reliable tool for assay development.

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DISCLOSURES:

The authors have nothing to disclose.

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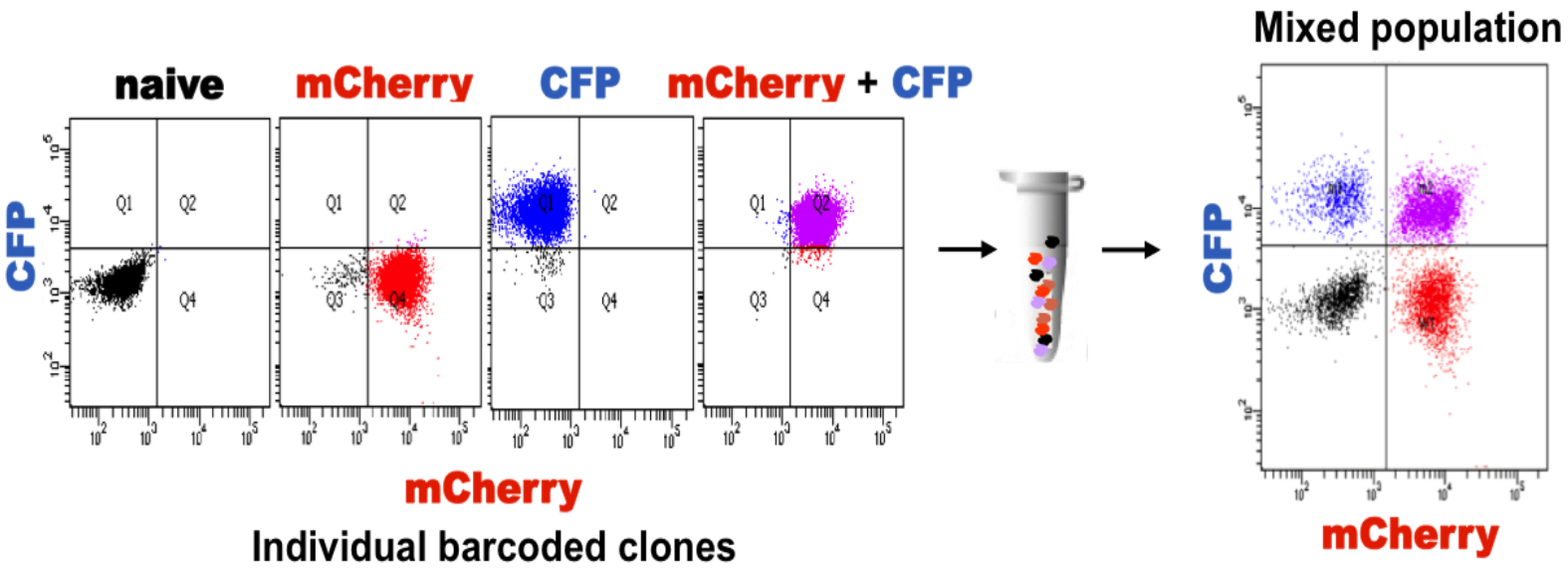

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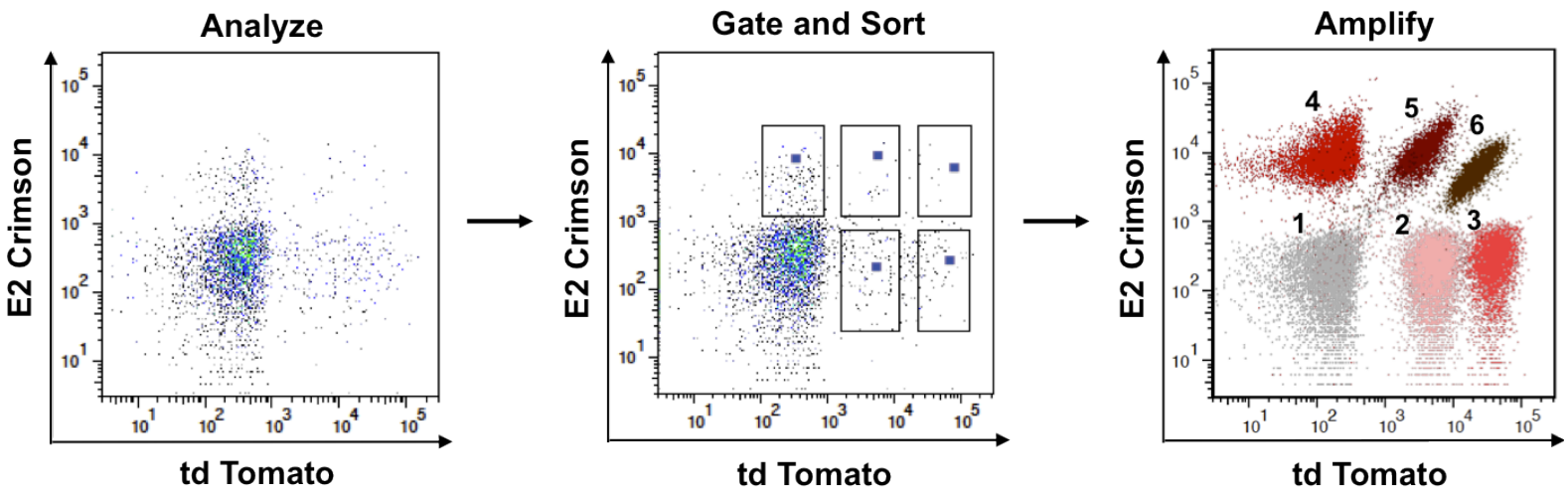

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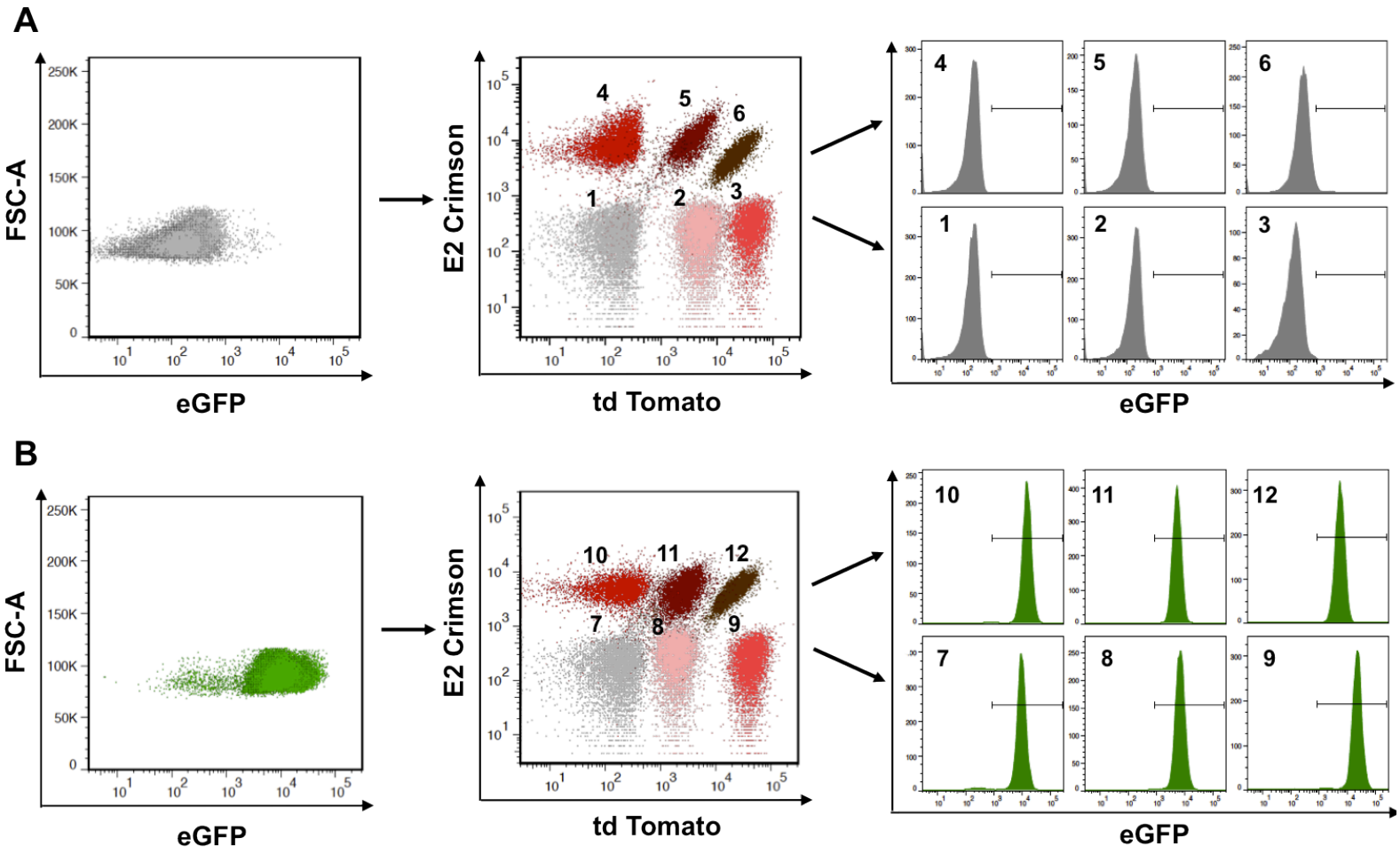

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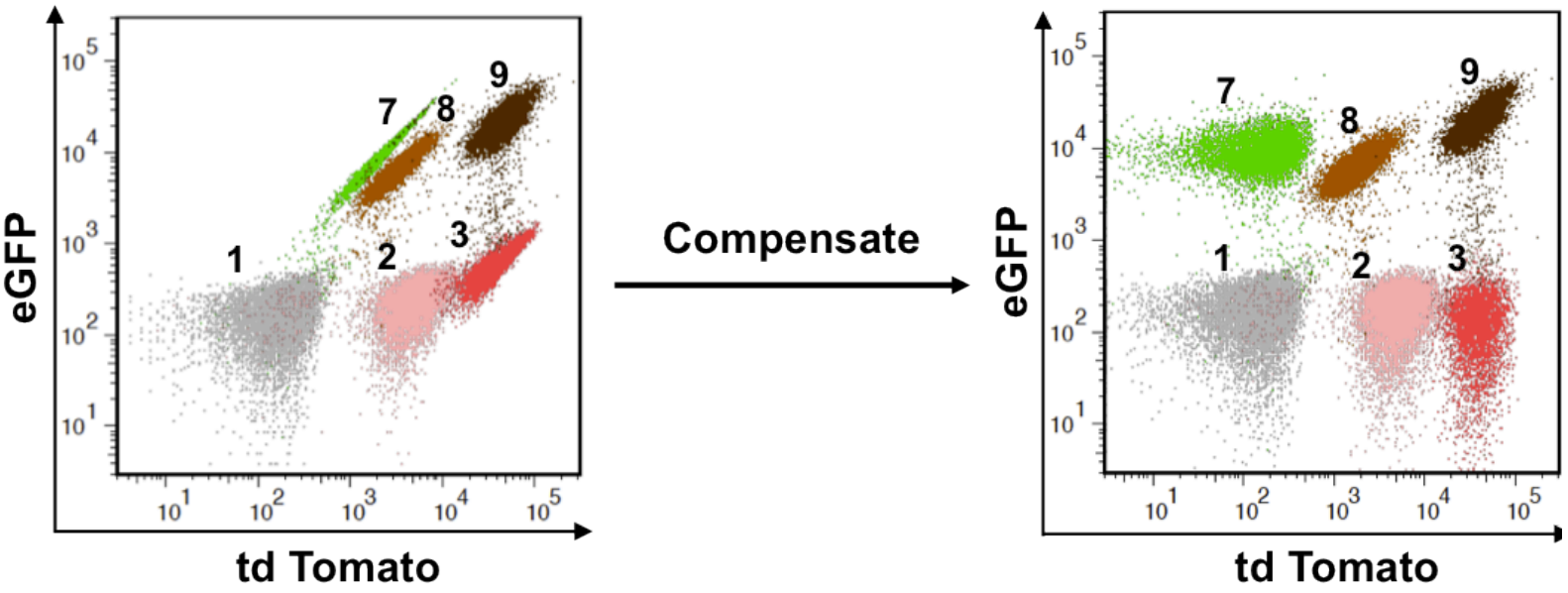

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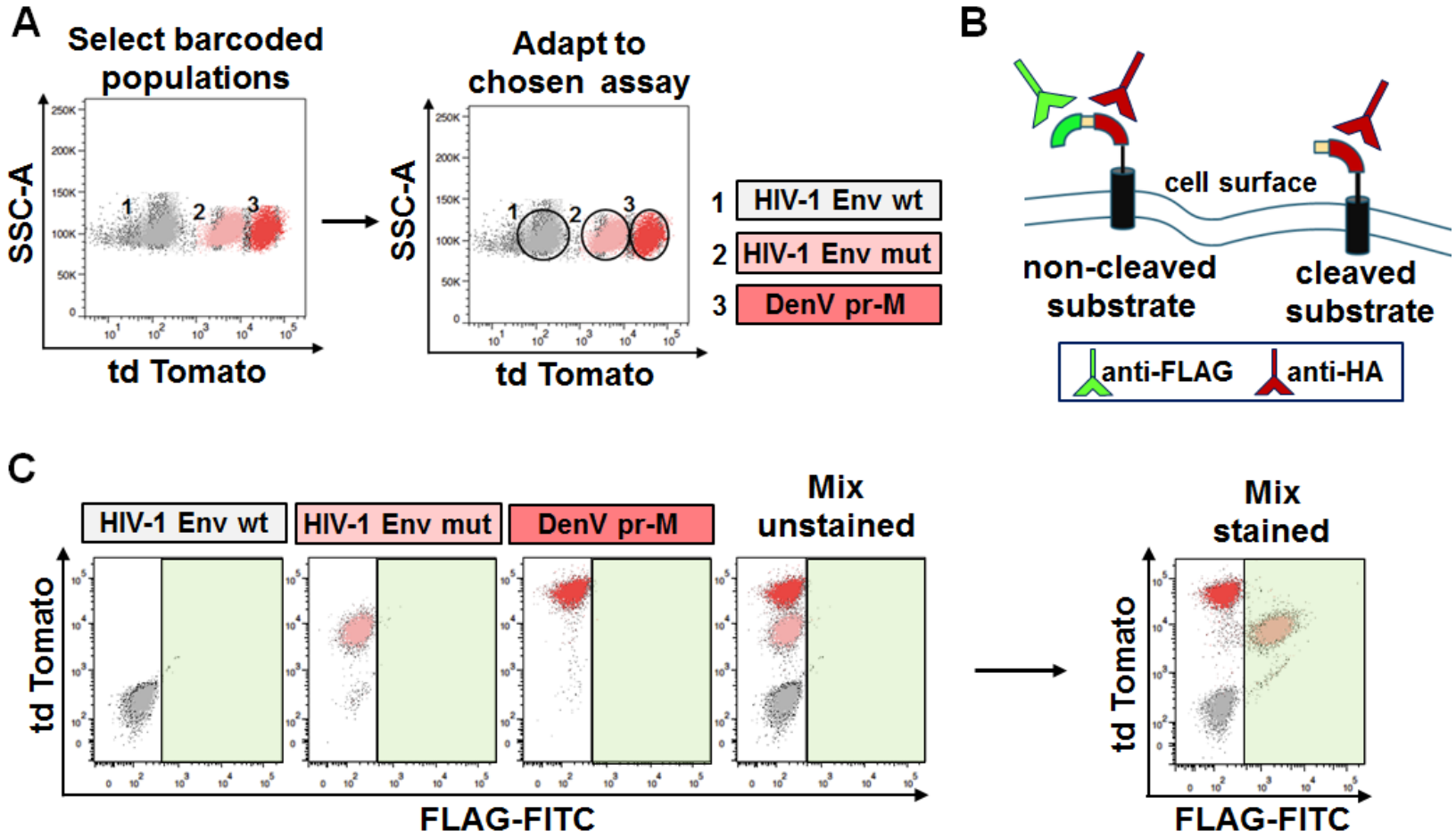

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Name of Material/ Equipment	Company	Catalog Number
10mL syringes	BD	309604
0.45µm plytetrafluoroethylen filter	pall corporation	4219
DMEM (Dulbecco's Modified Eagle Medium)	Corning	45000-304
PEI (Polyethylenimine)	poly sciences	23966-2
Hanging bucket centrifuge (refrigerated)	Eppendorf	5805 000.017
PBS (phosphate buffered saline)	Corning	21-040-CV
Polybrene (hexadimethreen bromide)	Sigma-Aldrich	107689
FACSAria	BD Biosciences	
FACSCanto	BD Biosciences	
Phoenix-GP	Gift from Gary Nolan	
Fetal calf serum	Mediatech	MT35015CV
SupT1 cells	ATCC	CRL-1942
HEK 293T cells	ATCC	CRL-11268
RPMI 1640	Corning	10-040-CV

Comments/Description
used for filtering the virus
used for filtering the virus
cell growth media for HEK 293T cells
2mg/mL concentration used
used for spin infection
used for washing of cells
Used to increase viral infection efficiency. Used at a 5µg/mL concentration.
instrument used for sorting cell populations
instrument used for cell analysis
cell line used to produced retroviral particles
used for cell growth and sorting
Human T lymphoblasts
Human Embryonic Kidney cells that also contain the SV40 large T-antigen
cell growth media for SupT1 cells

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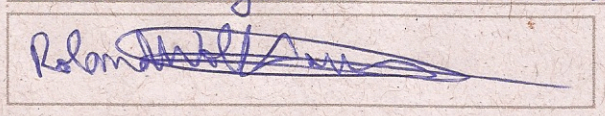
Signature: 

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List of changes based on Editorial and Reviewers comments
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List of changes based on Editorial comments:

We have answered, point by point, all comments of editor and reviewers below:

1. We retrieved the most recent version of the manuscript from the ‘file inventory’.

2. Short Abstract does not exceed 50 words.

3. We have included a step on how to prepare the mixture (step 1.2). We added an extra step (step 1.3) and a note regarding formation of polyplexes.

4. In step 1.6 (now 1.7) we have clarified that the solution is added to the plated cells.

5. We have simplified the protocol steps so that they are no more than 3-4 lines of text covering 2-3 actions. We have placed each "Note" on its own separate line. Highlighted text does not exceed 2.75 pages.

6. We have edited Figure 1 and Figure 5, which were substituted in this last resubmission.

List of changes based on Reviewers comments:

Reviewer #1:
Minor Concerns:

1. Introduction: We have deleted the definition of fluorescence.

2. In Figure 1, we have corrected the mislabeled axis.

3. Step 3.1: We have defined a "tight" population in terms of CV.

4) In order to further clarify the substrate assay, we have added a schematic of the assay in Figure 5 (now Figure 5b), edited the legend of the figure and edited the description in the results, which should help interpret the results.

Reviewer #2:
Major Concerns:
The major issue raises the lack of description regarding compensation and flow-cytometry technology. We feel this is not a ‘cytometry only’ based method. We agree with the reviewer that a reference or two needed to be added to guide the reader to the appropriate source for further reading on cytometry. We did add an explanation about compensation in the Discusssion (line 530).

Minor Concerns:
1. We have edited all text to make it less ‘ idiosyncratic’.

2. We have included in the discussion the usage of bar-coding for other fluorescence-based assay technologies such as high-content imaging.

Reviewer #3:

Major Concerns:

The reviewer raises a major concern regarding the use of one fluorescent marker expressed utilizing retroviral technology. It feels like the reviewer does not believe that retroviral transduction can produce a reliable source of cells for this goal, a statement we politely disagree with. Our expertise with this technology proves otherwise. The reviewer also mentions that we should focus on optimizing the transduction protocol for simultaneous expression of multiple fluorescent reporters, rather than a single marker at different intensities. While we have mentioned the utility of different fluorescent markers, we have decided to focus and explain in this protocol the use of one marker at different intensities. We have mentioned different markers in step 1.2 and 1.4. We have also mentioned in the first paragraph of the results (line 393-403) that one can utilize different markers rather than one at different intensities. Figure 1 also shows two different markers. Following figures (2 through 4), although they do show one of the markers at two intensities, also include an additional marker as well. See also lines 505-509 in the discussion. Finally, the reviewer mentions that expansion of the protocol in primary cells would significantly improve the quality and usefulness of this work. We feel that this is beyond the scope of the manuscript and protocol. Moreover, retroviral technology and selection of clones in primary cells is cumbersome, expensive and biologically difficult if not impossible to achieve, as descendant of these primary cells will lose their 'primary' properties.

Specific Comments and Minor Concerns:

1. We have commented on the usage of primary cells (see above).

2. We have described the optimal confluency for transfection and described the health of the cells on the note below step 1.1.

3. Line 158: We have removed the portion about lentivirus production.

4. Line 160 (now 164): Information about the packaging Phoenix-GP cell line was added, now in a new note.

5. Line 163 (now 168): We have addressed the questions regarding addition of the transfection mixture and clarified the steps. We added a note regarding the replacement of fresh media.

6. Line 169 (now 187): The reviewer raises lack of clarity regarding number of vectors for transfection. We have clarified this point within the two notes following step 1.2 and 1.6. Please be aware that verbiage within the original step 1.3 was moved to the note below step 1.6, decreasing the number of steps. Information regarding packaging constraints was also added.

7. Line 173 (now 192): The reviewer mentions that prior to the filtration through a 0.45μM membrane, an initial centrifugation step should be performed to pellet any non-adherent cells and/or debris. While we have never done this, such a note can be added if requested.

8. Line 180-181 (now 198): We have added a range for the number of cells used for transduction. We also added a note below step 1.5 describing the confluency for

adherent cells.

9. Line 184 (now 209): We have mentioned percentage infection rate rather than volume of viral supernatant and left the volume used as example. We also added a note below step 1.6 describing this. Please note that original step 1.6 was divided into two steps 1.6 and 1.7, to adhere to the two-three sentences rule per step.

10. Line 185 (now 208): We have clarified the addition step of polybrene.

11. Lines 232-269 (287-327): The reviewer has doubts as of the ability of retroviral transduction to produce cell lines stably expressing a fluorescent marker at different intensities. We have a well-established experience in doing so and published in Cytometry part A how these cell lines are stable and distinguishable for at least a period of six months. We feel it is beyond the scope of this methods paper to describe the scientific reason behind the difference in expression level utilizing retroviral technology. The matter of fact is that the technology, followed by cell sorting, does allow to purify distinguishable populations. But we did add, to ease the reviewer's point, a sentence in the discussion to clarify this point. We would like to remind that the last two sentences of the intro stated: 'This protocol is thus not intended to show the well-established retroviral/lentiviral technology for genetic transfer or the value of fluorescent proteins but rather to show the enhancing power of combining both for multiplexed applications'.

12. Lines 441-443 (now 459-462): Same point as the one above.

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