FROM A STEM CELL PERSPECTIVE

The technical challenges of working with stem cells can be daunting. Whether you are maintaining a population of undifferentiated stem cells or examining a specific differentiation pathway, the fast-paced arena of stem cell research relies on constantly adapting and improving the available cell biology tools. In this group of articles, we describe three fluorescence-based cell biology probes and their application to stem cell research. The Qtracker® Cell Labeling Kits are useful for labeling and subsequently separating feeder cells from stem cells by flow cytometry. The ELF® 97 Endogenous Phosphatase Detection Kit provides a simple method for monitoring alkaline phosphatase activity, a key marker of undifferentiated stem cells. And the LipidTOX™ neutral lipid stains selectively label neutral lipids in cells, enabling real-time detection and quantitation of adipogenesis. While these fluorescent probes have little in common structurally or mechanistically, they each have been shown to effectively address a specific facet of stem cell research.

Sorting out feeder cells co-cultured with embryonic stem cells

IDENTIFY AND SEPARATE INDIVIDUAL CELL TYPES USING QTRACKER® CELL LABELING KITS.

Technical barrier: Separating stem cells from their feeder cells

Human embryonic stem (hES) cells require culture under conditions that prevent differentiation. To maintain their potency, hES cells are commonly co-cultured with mitotically inactive mouse embryonic fibroblasts (MEF) as feeder cells. This practice, however, presents challenges to the subsequent physical isolation of hES cells, where MEF contamination is not desired. An effective cell separation strategy would utilize a long-lasting live-cell label that does not compromise viability yet provides a detectable handle for subsequent identification and isolation of labeled cells before or after fixation.

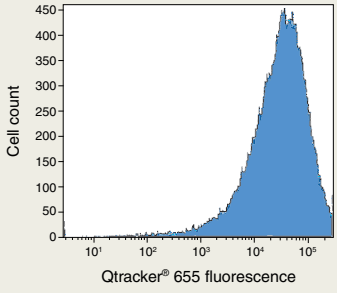

Figure 1—Flow cytometric analysis of mouse embryonic fibroblasts labeled with the Qtracker® 655 Cell Labeling Kit. Mitotically inactive mouse embryonic fibroblasts (MEF) were cultured for 48 hours and then labeled with the Qtracker® 655 Cell Labeling Kit. This FACS histogram illustrates that 97% of the MEF were labeled with the Qdot® 655 nanocrystals.

Solution: Labeling feeder cells with Qdot® nanocrystals

Qtracker® Cell Labeling Kits provide a simple and efficient method for fluorescently labeling MEF feeder cells with Qdot® nanocrystals, allowing straightforward identification and isolation of these cells in an hES cell co-culture. To gain access to the cytoplasm, the Qdot® reagents supplied in these kits employ a selective targeting peptide noncovalently bound to the fluorescent nanocrystal. Once internalized by the cell via endocytosis, the Qdot® nanocrystals exhibit intense, photostable fluorescence that can be observed using continuous illumination without time constraints due to photobleaching or degradation. The Qdot® nanocrystals are distributed in vesicles throughout the cytoplasm, can be observed up to five weeks after labeling, and are not transferred to adjacent cells in the population. Moreover, experiments indicate that Qtracker® labeling does not significantly affect cell viability or cellular enzyme activity.

We have demonstrated the utility of this cell separation strategy by labeling MEF using the Qtracker® 655 Cell Labeling Kit and then culturing these feeder cells with either SA2p12 hES cells or BG1vp22 hES cells. Our analysis of MEF labeled with Qdot® 655 nanocrystals by flow

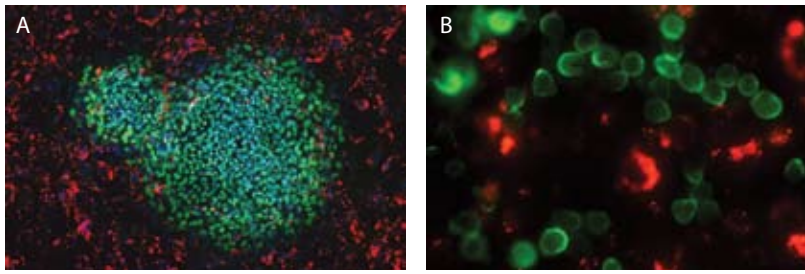

Figure 2—Immunocytochemical analysis of human embryonic stem (hES) cells co-cultured with mouse embryonic fibroblasts (MEF). BG1vp22 and SA2p12 hES cells were each plated onto a Qdot® 655 nanocrystal–labeled MEF feeder layer and allowed to grow for 48 hours. The cells were then fixed, labeled with anti-Oct4 or anti–Tra-1-81 primary antibody and green-fluorescent Alexa Fluor® 488 secondary antibodies, and analyzed by fluorescence microscopy. Micrograph A shows a colony of Oct4-expressing BG1vp22 cells (green) co-cultured with Qdot® 655 nanocrystal–labeled MEF (red) and counterstained with DAPI (blue). Micrograph B shows a suspension of Tra-1-81–expressing SA2p12 cells (green) co-cultured with Qdot® 655 nanocrystal–labeled MEF (red).

cytometry shows a labeling efficiency of 97% using the reagents and standard protocol provided by the kit (Figure 1). After co-culturing the labeled MEF with hES cells for 48 hours, the cell preparations were fixed and subjected to immunocytochemical analysis using primary antibodies specific for hES cell markers and secondary antibodies labeled with the green-fluorescent Alexa Fluor® 488 dye. For all markers tested, the labeled MEF were easily discriminated from colonies of BG1vp22 hES cells (Figure 2A) and from suspensions of SA2p12 hES cells (Figure 2B) using fluorescence microscopy. In fact, the hES cells appeared to exclude the feeder cells rather than grow on top of them. Furthermore, we obtained a clean separation of antibody-labeled hES cells and Qdot® 655 nanocrystal–labeled MEF by flow cytometry, with very little overlap of the two populations and no double staining observed in any of the cells (Figure 3).

These findings illustrate the usefulness of Qtracker® Cell Labeling Kits for labeling live feeder cells and subsequently separating them from co-cultured hES cells. Qtracker® Cell Labeling Kits are available containing Qdot® nanocrystals in one of seven brilliant fluorescent colors (525 nm, 565 nm, 585 nm, 605 nm, 655 nm, 705 nm, or 800 nm emission) and can be used together for multiplexing applications. ■

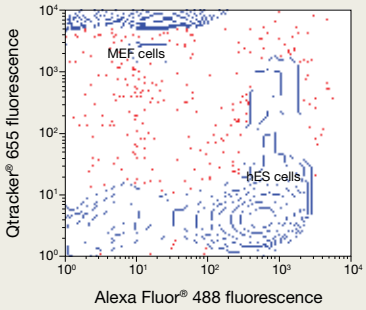

Figure 3—Flow cytometric analysis of human embryonic stem cells and mouse embryonic fibroblasts. Human embryonic stem (hES) cells, co-cultured with Qtracker® 655 nanocrystal–labeled mouse embryonic fibroblasts (MEF), were labeled with anti-SSEA4 primary antibody and Alexa Fluor® 488 secondary antibody. The cells were then analyzed by flow cytometry using 488 nm excitation. This contour plot, gated on cells by scatter, shows Alexa Fluor® 488 fluorescence vs. Qtracker® 655 fluorescence. The cultured hES cells show both positive and negative populations for SSEA-4 expression and have low fluorescence in the Qtracker® 655 channel. The MEF cells are so much brighter than the hES cells in the Qtracker® channel that both populations could not be on scale at the same time. This population resolution allows for easy discrimination or sorting of MEF from hES cells.

Multiplexing your panel of embryonic stem cell markers

LABEL UNDIFFERENTIATED CELLS USING ELF® 97 ALKALINE PHOSPHATASE SUBSTRATE.

Technical barrier: Finding compatible labels for multiplex assays

Endogenous alkaline phosphatase expression is a well-established marker for undifferentiated embryonic stem cells. In this specialized application, however, traditional histological methods for alkaline phosphatase fall short. Colorimetric and fluorometric reagents, including the fast red violet dye, can generate high background signals and poor resolution. More importantly, these traditional histochemical stains are generally not amenable to assays designed for detecting the expression of multiple markers in a single cell preparation. An alkaline phosphatase–selective stain that can be multiplexed with other embryonic stem cell markers would provide significant advantages, not only for the characterization of embryonic stem cell lines, but also for the general investigation of the role of alkaline phosphatase in embryonic, developmental, and pathological pathways. →

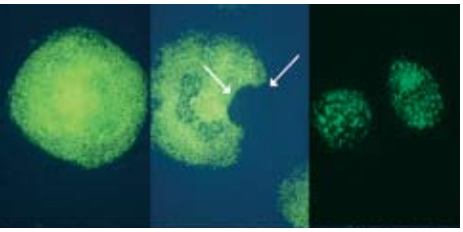

Figure 4—Human embryonic stem cells labeled with ELF® 97 alkaline phosphatase substrate. BG01V human embryonic stem (hES) cells (ATCC® SCRC-2002™) surrounded by feeder cells are fully undifferentiated, as evidenced by the presence of continuous green ELF® 97 fluorescence throughout the cell mass (left). While most of this BG01V colony remains undifferentiated, a locus of cells has differentiated, as shown by the lack of ELF® 97 fluorescence in one region (middle, arrows). The uneven green ELF® 97 fluorescence in these R1/E mouse embryonic stem cells (ATCC® SCRC-1036™) indicates nonuniform maintenance of the undifferentiated state (right). Images contributed by the American Type Culture Collection (ATCC®).

Solution: Multiplexing stem cell markers with ELF® 97 phosphate

ELF® 97 phosphate is a state-of-the-art fluorogenic phosphatase substrate that exhibits several key attributes for alkaline phosphatase localization in cells and tissues. Upon enzymatic cleavage, this weakly blue-fluorescent substrate yields a bright yellow-green–fluorescent precipitate with an unusually large Stokes shift and extremely photostable fluorescence (up to 500 times the photostability of fluorescein). These unique spectral characteristics make ELF® 97 phosphate optimally suited for multiparameter fluorescence assays.

Because the fluorescence excitation and emission maxima of the ELF® 97 precipitate (365/530 nm) are well separated, you can easily resolve the ELF® 97 signal from autofluorescence as well as from the signals of fluorescent antibody conjugates, binding proteins, and cellular counterstains. For example, ELF® 97 fluorescence emission can be clearly distinguished from that of the green-fluorescent Alexa Fluor® 488 dye by fluorescence microscopy using a standard DAPI longpass filter.

Furthermore, most phosphatase substrates used in histochemistry yield colorless, soluble hydrolysis products that must be coupled with a capture reagent to generate a colored or fluorescent precipitate. In contrast, hydrolysis of ELF® 97 phosphate generates a fluorescent precipitate at the site of enzymatic activity, which not only leads to higher resolution and lower background but also significantly reduces the amount of time required for detection.

Because endogenous alkaline phosphatase expression is a well-established marker for undifferentiated embryonic stem cells, Plaia and coworkers recently reported the use of the versatile ELF® 97 Endogenous Phosphatase Detection Kit to characterize a new variant human embryonic stem cell line, BG01V[1] (Figure 4). ELF® 97 phosphate was found to be so useful in this application that ATCC® (American Type Culture Collection) licensed the ELF® 97 technology and developed an optimized protocol and kit specifically designed for embryonic stem cell applications. The ATCC® ELF® Phosphatase Detection Kit (cat. no. SCRR-3010, www.atcc.org) is easy to use and signal development is very rapid, usually only 30–90 seconds. ■

Following differentiation into adipocytes

STUDY ADIPOGENESIS USING LIPIDTOX™ NEUTRAL LIPID STAINS.

Technical barrier: Selectively detecting neutral lipids

Adipogenesis, or the differentiation of preadipocytes into fat cells, provides a window into the complex sequence of events that directs multipotent progenitors to undergo phenotypic changes upon differentiation.[2] Implicated in several diseases including obesity, atherosclerosis, and steatosis,[3] the adipogenic pathway is actively studied using cellular models such as mouse 3T3-L1 fibroblasts and human mesenchymal stem cells in order to better understand and regulate this process.

Nile red, the standard reagent for monitoring neutral lipid accumulation during adipogenesis,[4] has significant drawbacks in this application. Importantly, nile red is not a specific stain for neutral lipids because it also stains phospholipids. Moreover, the fluorescence emission spectrum of nile red is quite broad, spanning the green (FITC) and red (TRITC) channels and complicating multiparametric studies. A neutral lipid–specific fluorescent stain with a narrow emission spectrum would allow researchers to better target the accumulation of fat droplets during adipogenesis and to combine this labeling with other cell markers.

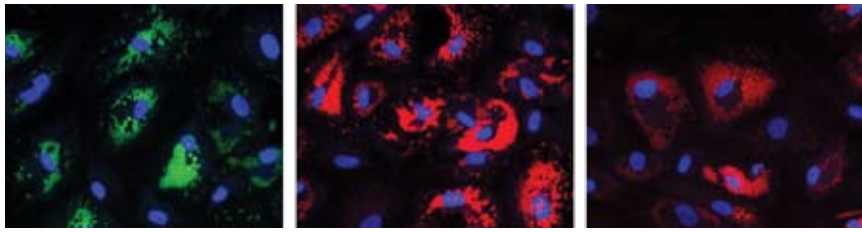

Figure 5—LipidTOX™ neutral lipid staining of lipid droplets in adipocytes. Adipocytes differentiated from human mesenchymal stem cells were fixed with formaldehyde and then stained with LipidTOX™ Green (excitation/emission ~495/505 nm) (left), LipidTOX™ Red (excitation/emission ~577/609 nm) (middle), and LipidTOX™ Deep Red (excitation/emission ~637/655 nm) (right) neutral lipid stains (green, bright red, and dark red lipid droplets, respectively); nuclei were counterstained with DAPI (blue).

Solution: Examining adipogenesis with LipidTOX™ neutral lipid stains

Originally developed for high content screening (HCS)–based cytotoxicity assays, LipidTOX™ neutral lipid stains are very useful for studying adipogenesis. Now available as stand-alone reagents, LipidTOX™ neutral lipid stains provide a simple, rapid, and specific method for staining neutral lipids in live or fixed cells. These neutral lipid–specific fluorescent dyes—available with narrow green, red, or far-red emission (Figure 5)—are supplied as ready-to-use formulations for easy setup, and require no sonication or organic solvents before use nor wash steps prior to imaging. Furthermore, the LipidTOX™ neutral lipid stains are readily incorporated into standard cell labeling procedures, including immunocytochemistry protocols, provided that detergents such as saponin or digitonin are used for cell permeabilization (Figure 6).

LipidTOX™ Green, Red, and Deep Red neutral lipid stains have been validated in studies with cells undergoing adipogenesis, as well as in cytotoxicity assays with compounds known to cause steatosis. With their selective labeling and narrow fluorescence emissions, these LipidTOX™ stains provide researchers with the specificity and flexibility needed for multiparametric analysis in adipogenesis applications. The LipidTOX™ Green neutral lipid stain is also available as part of the HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit, which provides a complete set of reagents for performing image-based HCS assays to characterize the potentially toxic side effects of compounds on lipid metabolism in mammalian cell lines. ■

References

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2. Rosen, E.D. and Spiegelman, B.M. (2000) *Annu Rev Cell Dev Biol* 16:145–171.
3. Gregoire, F.M. et al. (1998) *Physiol Rev* 78:783–809.
4. Greenspan, P. and Fowler, S.D. (1985) *J Lipid Res* 26:781–89.

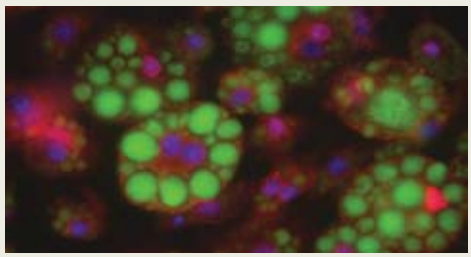

Figure 6—LipidTOX™ Green neutral lipid stain and fatty acid–binding protein (FABP4) antibody labeling in adipocytes. Adipocytes differentiated from 3T3-L1 mouse fibroblasts were fixed with formaldehyde and permeabilized with saponin before labeling with rabbit IgG anti–fatty acid binding protein (FABP4) antibody and Alexa Fluor® 594 goat anti–rabbit IgG antibody (red). These cells were then stained with LipidTOX™ Green neutral lipid stain (green), counterstained with DAPI (blue), and mounted in ProLong® Gold antifade mounting medium.

Product	Quantity	Cat. no.
Qtracker® 525 Cell Labeling Kit	1 kit	Q25041MP
Qtracker® 565 Cell Labeling Kit	1 kit	Q25031MP
Qtracker® 585 Cell Labeling Kit	1 kit	Q25011MP
Qtracker® 605 Cell Labeling Kit	1 kit	Q25001MP
Qtracker® 655 Cell Labeling Kit	1 kit	Q25021MP
Qtracker® 705 Cell Labeling Kit	1 kit	Q25061MP
Qtracker® 800 Cell Labeling Kit	1 kit	Q25071MP
ELF® 97 Endogenous Phosphatase Detection Kit	1 kit	E6601
ELF® spin filters *20 filters*	1 box	E6606

Product	Quantity	Cat. no.
HCS LipidTOX™ Green neutral lipid stain *solution in DMSO* *for cellular imaging*	each	H34475
HCS LipidTOX™ Red neutral lipid stain *solution in DMSO* *for cellular imaging*	each	H34476
HCS LipidTOX™ Deep Red neutral lipid stain *solution in DMSO* *for cellular imaging*	each	H34477
HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit *for high content screening* *for cellular imaging* *2-plate size*	1 kit	H34157
HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit *for high content screening* *for cellular imaging* *10-plate size*	1 kit	H34158