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Dual Transgene Strategy for Live Visualization of Chromatin and Plasma Membrane Dynamics in Murine Embryonic Stem Cells and Embryonic Tissues

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Summary

To simultaneously follow multiple subcellular characteristics, for example, cell position and cell morphology, in living specimens requires multiple subcellular labels. Toward this goal, we generated dual-tagged mouse embryonic stem (ES) cells constitutively expressing differentially localized, spectrally distinct, genetically encoded fluorescent protein fusions. We have used human histone H2B fusions to fluorescent proteins to mark chromatin. This provides a descriptor of cell position, division, and death. An additional descriptor of cell morphology is achieved by combining this transgene with select lipid-modified fluorescent protein fusions that mark the plasma membrane. Using this strategy, we were able to live image cellular dynamics in three dimensions over time both in cultured ES cells and in mouse embryos generated using dual-tagged ES cells. This study, therefore, presents the feasibility of applying multiple spectrally and subcellularly distinct fluorescent protein reporters for live imaging studies in ES cells and mouse embryos. Furthermore, the increasing availability of spectral variant fluorescent proteins along with the development of methods that permit improved spectral separation now facilitate multiplexing of fluorescent reporters to provide readouts of a variety of anatomical and physiological behaviors simultaneously in living specimens.

Keywords

live imaging; 3D time-lapse; confocal; fluorescent proteins; GFP; RFP; mCherry; embryonic stem cells; mouse embryo; chimera

A sequence of stereotypical morphogenetic movements plays a pivotal role in building the developing embryo. A goal of developmental biology is to elucidate the cellular and molecular interactions that co-ordinate the specification of different cell types and the establishment of body plans, and to understand these interactions in the context of the global morphology of the embryo. Cell behaviors and motions in a living system are often complex and cannot be inferred with great accuracy from observations of static, sequentially staged images.

The demonstration that green fluorescent protein (GFP) was amenable to use in heterologous systems has spearheaded a revolution in live imaging technology (Chalfie *et al.*, 1994). Various bright, photostable, and developmentally neutral fluorescent proteins (FPs) with unique spectral properties are now available including enhanced GFP (EGFP) and its spectral variants (Heim *et al.*, 1994; Heim and Tsien, 1996; Nagai *et al.*, 2002). These variants include enhanced

cyan FP (ECFP) and Venus [a bright yellow (Y)FP variant], in addition to red FPs (RFPs), which include mRFP1, the first monomeric DsRed derivative, and its variant mCherry (Campbell *et al.*, 2002; Shaner *et al.*, 2004). Advances in optical imaging modalities and the continued evolution of these genetically encoded FPs is facilitating the study of cell behavior at high temporal and spatial resolution in living samples. Thus, coupled with genetics, live imaging and genetically encoded reporter strains represent an essential platform for acquiring quantitative information on dynamic cell behaviors and cell fate in vivo.

Histological stains have traditionally been limited in functionality by the ability of the human eye to resolve multiple colors. In more recent years, the availability of a wide array of FPs with emission spectra exceeding the breadth of visible wavelengths has provided an alternative to traditional chromogenic stains. It is now possible that routine histological analyses, for example using hematoxylin and eosin for staining nuclei and cell morphology, respectively, in tissue sections, might be superseded by methodologies providing equivalent information, but in three dimensions (3D), or in 3D over time (3D time-lapse) yielding dynamic information on intrinsic cell behaviors operating in living specimens. To this end, we have previously described the generation of developmentally neutral FP reporters in which EGFP or Venus were fused to human histone H2B to label the nucleus providing a descriptor of cell position, division, and death, or lipid-modified providing a descriptor of cell morphology (Fraser *et al.*, 2005; Hadjantonakis and Papaioannou, 2004; Rhee *et al.*, 2006). These tools provide the basis for the current study, which sought to combine these reporters for increased resolution and information content live imaging applications.

Among the unrealized goals of live imaging technology is the idea that nearly all components and processes of a cell could be labeled and visualized simultaneously to generate a complex 4D understanding of cellular dynamics in a complex cell population or in an organismal context (Hadjantonakis *et al.*, 2003; Megason and Fraser, 2003; Passamaneck *et al.*, 2006). In practice, this dream has been grounded by the available genetically encoded FP reporters, the technologies for excitation and detection of multiple fluorophores, and the ability to deliver these constructs to living tissues through transient or stable transgenesis.

Previous studies that sought to document multiply or differentially tagged cells in an organismal context have either relied upon widefield microscopy techniques to document samples (Yamamoto *et al.*, 2004) or have imaged samples at single time points (Livet *et al.*, 2007). While suitable for thin layers of cultured cells, laser scanning confocal excitation is preferable for generating data on thicker samples such as cellular colonies or embryos and affords the advantage of facilitating three-dimensional (3D) image information at time intervals. Recently, Digital Scanned Laser Light Sheet Fluorescence microscopy DLSM was reported as a compelling imaging modality for live cell imaging, creating an environment in which, among other advantages, zebrafish embryos were exposed low-energy environments that reduced the effects of phototoxicity. DLSM is currently unreported for other species or tissues, but contains optical solutions for many of the challenges typically faced when imaging the embryos of other common laboratory species (Keller *et al.*, 2008). Moreover, the advent of spectral analysis modalities accompanying the latest generation of confocal microscopes (Hadjantonakis *et al.*, 2003; Zimmermann, 2005; Zimmermann *et al.*, 2003) has allowed for the separation of closely overlapping spectra (Dickinson *et al.*, 2003; Livet *et al.*, 2007; Rhee *et al.*, 2006). This applicability has been demonstrated using up to four spectrally distinct proteins electroporated into the neural tube lumen of chick embryos (Teddy and Kulesa, 2004; Teddy *et al.*, 2005), or three distinct proteins visualized in zebrafish embryos (Finley *et al.*, 2001). However, the invasive nature and low transfection efficiency of such experiments is a substantial drawback. Additionally, the experimenter has little control over the levels at which the transgenes are expressed in any sample. This may vary from weakly expressing cells to toxicities induced by high levels of transgene expression, to cells that express only a subset

of the fluorophores. Thus, reproducibility between experiments is not assured. While comparable experiments have yet to be reported in the mouse, it is, in some respect, a more favorable system for such studies. The mouse is the premiere mammalian genetic model and possesses a sequenced genome, which is readily manipulated by homologous recombination or random transgene integration. The ability to create genetic modifications that are encoded by the nuclear genome and are germline-transmissible circumvents many of the limitations described earlier.

Transgenes encoded by the nuclear genome are, however, generally expressed at lower levels than transient transfections, and are thus more difficult to visualize. The benefit, however, is that the expression levels are essentially consistent from experiment to experiment, and from animal to animal. To address these issues and to generate tools for high-resolution live imaging of cell morphology, division, position, and death in ES cells and mouse embryos, we generated dual-tagged ES cells expressing simultaneously fluorescent fusion proteins labeling the plasma membrane and the nucleus of the cell. We generated mouse embryonic stem (ES) cell lines stably expressing two spectrally distinct subcellular localized FPs, namely, histone H2B FP fusions labeling active chromatin (Hadjantonakis *et al.*, 2003; Hadjantonakis and Papaioannou, 2004) and lipid-modified FP fusions labeling the plasma membrane (Rhee *et al.*, 2006). All FPs were placed under the widespread CAGGS enhancer/promoter element, comprising the chicken β -actin promoter, first exon/intron, and CMV immediate early enhancer (Niwa *et al.*, 1991).

For optimal fluorescence intensity and spectral separation, we partnered a GFP with a RFP. We noted that an H2B-mRFP1 fusion (Passamaneck *et al.*, 2007) was toxic in ES cells and mice, and so opted to use an H2B-mCherry fusion alongside the green fluorescent H2B-GFP fusion for nuclear labeling, and one of two types of lipid-modified fusions, either incorporating a myristoylation sequence or a glycosylphosphatidylinositol (GPI) anchor, for directing RFP or GFP to the plasma membrane. The data presented depict GFP-GPI and myr-mRFP1 fusions; however, we noted that overall, both lipid-modifications gave comparable plasma membrane labeling, and that lipid-modified mRFP1 fusions were equivalent to mCherry fusions in their developmental neutrality.

Of note, RFPs are the FPs of choice for live imaging applications and much effort has gone into identifying and/or developing developmentally neutral RFPs (Campbell *et al.*, 2002; Shaner *et al.*, 2004; Shaner *et al.*, 2005). The longer wavelength emission and excitation spectra of RFPs provide several advantages. RFPs are less toxic for the cell due to their longer wavelength required for fluorophore excitation. Moreover, longer wavelengths penetrate deeper into the tissue and their spectra do not overlap with the widely used green, blue (cyan), and yellow FPs. Hence, the combination of red and green FPs is, in most cases, optimal for combinatorial imaging. YFP can be used instead of GFP in combination with RFP. In cases where only YFP or GFP can be imaged, and must be imaged in combination with CFP, linear unmixing can be used to give the necessary separation of signals. This involves the computational separation of their overlapping spectra, which alleviates signal bleed through (Zimmermann, 2005; Zimmermann *et al.*, 2003).

To obtain dual-tagged ES cell lines, we electroporated two reporter-expressing constructs, for example either H2B-mCherry and GFP-GPI or H2B-GFP and myr-mRFP1, into ES cells along with a circular plasmid encoding Puromycin drug resistance. To reduce the risk of cellular toxicity, dual-tagged ES cell clones, which expressed minimal readily detectable levels of fluorescence within the same dynamic range for each fluorophore were identified and expanded. Dual-tagged ES cell colonies were imaged with laser scanning confocal microscopy to reveal high contrast-specific labeling of sub-cellular compartments. In Figure 1, H2B-mCherry expression in the nucleus and GFP-GPI on the plasma membrane of the cell allow

us to readily distinguish the position and morphology of each cell within a multicellular ES cell colony.

These data highlight the use of subcellularly localized FP fusions as advantageous above the simple use of native FPs, which often prevent discrimination of closely opposed cells. The present FP configuration allows for individual cells to be resolved by identification of the nucleus, while also providing data on membrane architecture yielding information on cell morphology (see Fig. 1). Using such combinatorial reporters multinucleate cells (for example, myofibers or hepatocytes) may also be characterized (our unpublished observations). As noted previously with GFP-based H2B fusions, chromosomes were readily observed in dividing cells, which due to the condensation of chromatin also exhibited increased fluorescence. Thus analyses of cell cycle, death, and membrane uptake may all be made within the same sample. 3D time-lapse imaging of dual-tagged ES cells revealed dynamic cell behaviors including cell division, movement, and morphology changes in individual cells over time (see Fig. 2).

To determine if these dual tags were developmentally neutral and to investigate the resolution of data that could be obtained with them in mouse embryos, we generated chimeric embryos by ES cell aggregation. Chimeras containing H2B-mCherry, GFP-GPI or H2B-GFP, and myr-mRFP1 ES cells were then imaged at different developmental stages (see Fig. 3). Both H2B-mCherry, GFP-GPI and H2B-GFP; myr-mRFP1 ES cells routinely produced chimeras with a high level (>90%) of ES cell contribution (Fig. 3n-t) and full term development. We were able to dilute the ES cell contribution by aggregating reduced numbers (1–2 vs. 15) cells (Fig. 3g–l). Live imaging of chimeras at preimplantation (blastocyst), early postimplantation (E6.5) and midgestation (E9.5) stages, revealed that nuclei (a descriptor of a cell's position) and plasma membrane (a descriptor of a cell's morphology) were readily distinguishable (see Fig. 3).

Since these subcellular labels are developmentally neutral, they can be used to visualize different cell populations. Since limiting photon exposure of samples in live imaging applications helps maintain fidelity, minimizing the number of fluorophores helps meet this goal. This can be achieved by using the same fluorophore to label and acquire differential information in distinct cell types. Therefore, dual spectrally and subcellularly distinct labels afford a high-resolution binary color-code for live imaging such that multiple distinct populations can be visualized with a limited number of fluorophores. Using just two spectrally distinct (GFP vs. RFP) and differentially localized FPs (nuclear vs. plasma membrane), up to four different cell populations can be tagged. By live imaging an ES cell colony comprising two cell populations labeled with H2B-GFP; myr-RFP and H2B-mCherry; GFP-GPI, respectively, we could readily detect and discriminate between two populations of differentially coded cells (see Fig. 4).

In this study, we have shown the feasibility of using spectrally and subcellularly distinct FP reporters for live imaging at high-resolution in ES cells and in mouse embryos. We demonstrate that cell labeling with only two colored FPs gives us a powerful tool at hand to simultaneously analyze cell morphology, division, and positions. Furthermore, a binary code using only two spectrally distinct colors allows imaging of cell dynamics and discrimination of up to four different cell populations. This type of approach can readily be incorporated into transgenic and gene targeting regimes to direct line-age-specific transgene expression.

In addition, this methodology can be extended through use of more than two fluorophores to label different cells, as well as tagging additional subcellular compartments, such as the cytoskeleton by labeling actin or tubulin, and organelles such as mitochondria, as has been demonstrated in the chick embryo (Teddy *et al.*, 2005). In combination with live imaging subcellular tagging permits visualization of intracellular dynamics. Since generating multiple

independent transgenics in ES cells and mice is cumbersome, use of bi- or multicistronic vectors driving expression of multiple FP reporters through the use of an internal ribosome entry sites (IRES) or viral 2A peptide-based sequences (Osborn *et al.*, 2005; Szymczak and Vignali, 2005) should facilitate multiplex labeling, ensure expression in each FP fusion at stoichiometric levels and reduce the numbers of animals needed if these reporters are introduced into mutant strains. Though germline transmission has not been pursued for the constructs studied in this report, it should be noted that integrating DNA constructs, even constructs of heterologous sequences, typically do so at single loci (Brinster *et al.*, 1981).

In summary, live imaging combined with the power of mouse genetics represents the essential next step forward toward unraveling the dynamic mechanisms regulating embryonic development. This study demonstrates that spectrally distinct nuclear and plasma membrane localized FP reporters provide high-resolution live imaging information that is equivalent to, or goes beyond, the resolution of traditional histology. By applying this type of imaging tool, we can begin to probe the live cell behaviors directing normal mouse embryonic development and contrast with situations where development is perturbed.

METHODS

Plasmids *pCX::H2B-GFP*, *pCX::H2B-mRFP1* and *pCX::GFP-GPI* have been described previously (Hadjantonakis and Papaioannou, 2004; Passamaneck *et al.*, 2007; Rhee *et al.*, 2006). *pCX::myr-RFP* was generated by PCR of *mRFP1* from *pRSET-mRFP1* (Campbell *et al.*, 2002) using an oligo containing a N-myristoylation sequence (CTT GAA TTC GCC ACC ATG GGA AGC AGC AAG AGC AAG CCA AAG GCC TCC TCC GAG GAC GTC ATC AAG G) and a 3' oligo (CAA GC TTC GAA TTC TTA GGC GCC). The resulting PCR product was cloned into a *pCRII-TOPO* (Invitrogen) and the insert excised with *EcoRI* and cloned into *pCAGGS* (Niwa *et al.*, 1991). *pCX::H2B-mCherry* was generated excising *mCherry* from *pRSETmCherry* (Shaner *et al.*, 2004) using *BamHI* and *EcoRI*, an *EcoRI* and *NotI* adaptor oligo, and cloning into the *pCMV::H2B-GFP* vector (Hadjantonakis and Papaioannou, 2004) and exchanging *EGFP* for *mCherry*. *H2B-Cherry* was then cloned into *pCAGGS* (Niwa *et al.*, 1991) using *Xho* and *NotI*.

R1 ES cells were used for generating dual-tagged transgenic ES cells (Nagy *et al.*, 1993). Transgenic ES cell lines constitutively express H2B-GFP; myr-RFP or H2B-mCherry; GFP-GPI and were generated by co-electroporation of *ScaI*-linearized constructs and a circular *PGK-Puro-pA* plasmid conferring transient puromycin resistance (Tucker *et al.*, 1996). Puromycin selection was carried out as described previously (Hadjantonakis *et al.*, 1998; Long *et al.*, 2005). Fluorescent colonies were identified and picked under an epifluorescence stereo dissecting microscope. Clones were passaged in 96-well plates according to standard protocols (Nagy, 2003), and scored for the maintenance, subcellular localization, and level of fluorescence upon passage in culture in the absence of selection.

Aggregation chimeras were generated exactly as previously described (Eakin and Hadjantonakis, 2006). Embryos were dissected in DMEM/F12 (GIBCO) and 5% Newborn Calf Serum (Cambrex). For vibratome sections, embryos were either sectioned unfixed or fixed in 4% paraformaldehyde over night, washed 3× in PBS. Embryos were embedded in 5% sucrose; 4% low melting point agarose in PBS. Blocks were trimmed using a scalpel blade, and mounted onto a vibrating microtome (Leica VT1000) chuck using superglue. Sections were cut at a thickness of 50–200 μm.

Live ES cells and embryos were imaged in coverslip-bottomed dishes (MatTek). Samples were maintained in tissue culture media (ES cells) or 50% rat serum; 50% DMEM/F12 (embryos) under physiological conditions in a temperature-controlled, humidified chamber (Solent Sci,

UK) and a 5% CO₂ ; 95% air atmosphere. Laser scanning confocal data was acquired in using a Zeiss LSM510 META on a Zeiss Axiovert 200M. Raw data was processed using Zeiss AIM software (Carl Zeiss Microsystems at <http://www.zeiss.com/>), and Adobe Photoshop CS2 (Adobe Systems, San Jose, CA).

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FIG. 1.

Live imaging of ES cells expressing a GFP fusion and a RFP fusion labeling the plasma membrane and the nucleus, respectively. Live laser scanning confocal imaging of GPI-GFP and mH2B-Cherry dual-tagged ES cells providing information on cell morphology, cell position, and division. Schematic representation of ES cell colony (a). Red line depicts level of section represented in b–d of a z-stack taken from an ES cell colony. 2D images of single optical sections taken at a depth of 6 μm taken from a z-stack (b–f). Schematic representation of ES cell colony (g). Red line depicts level of z-section represented in h–l. 2D images of single optical sections taken at a depth of 11 μm taken from the same z-stack (h–l). 3D-rendered images of the entire z-stack of 32 xy-sections taken at 1 μm intervals (m–q). Brightfield images of single sections and the 3D reconstruction, respectively (b, h, and m). Images of green channel only (c, i, and n). Images of red channel only (d, j, and o). Merge of green and red channels (e, k, and p). Overlays of green and red channels onto brightfield images (f, l, and q). Scale bar: 20 μm .

FIG. 2.

Dual color 4D time-lapse imaging of dual-tagged embryonic stem cells showing dynamic cell movement and cell divisions. A population ES cells expressing the fluorescent fusion proteins GPI-GFP and H2B-mCherry are depicted in a series highlighting changes in cell morphology and cell divisions over time. 2D representations of single channel fluorescence images of green (a–f) and red (g–l) channels only showing dynamic changes in cell morphology and the cell nucleus, respectively. 2D merge of the green and red fluorescent channels (m–r). 3D reconstruction of the time-lapse sequence representing green and red channels (s–x). Scale bar: 20 μm .

FIG. 3.

Live imaging of embryos containing dual-tagged cells. Mouse chimeras generated using H2B-mCherry; GFP-GPI dual-tagged ES cells were live imaged using a laser scanning confocal microscopy. Chimeric blastocysts containing dual-tagged cells within the inner cell mass (**a–f**). Chimeric E6.5 embryo (**g–l**). Vibratome section of a chimeric E9.5 embryo (**n–t**). Bright field images (**a**, **g**, and **q**). Wholemount view of an E9 embryo (**m**). Dashed red line indicates the level of section shown in **n–t**. Single optical sections in single fluorescence channels showing GFP expression (**b**, **h**, **n**, and **r**) and RFP expression (**c**, **i**, **o**, and **s**) in dual-tagged cells of chimeric embryos. Merged images of green and red fluorescent channels (**d**, **j**, **p**, and **t**). Overlay of green and red channels onto bright field image (**e** and **k**). 3D-rendered images of *z*-stacks taken from the blastocyst and the E6.5 embryo, respectively (**f**, **l**). 3D-rendered images of E9 vibratome sections (**n–p**, **r–t**). High magnification view (**q–t**) of boxed region in **n–p** showing dual-tagged cells in the ectoderm of the otic vesicle as well as in the adjacent neural tube. Scale bars: 20 μ m (**f**), 50 μ m (**l**), and 200 μ m (**p**).

FIG. 4.

Live imaging binary color-coded colonies of dual-tagged ES cells. Schematic representation of a binary color code that can be used in cells to distinguish different populations. Using only two spectrally distinct FPs tagged to two spatially distinct locations (the plasma membrane and the nucleus), up to four different cell populations can be identified (only two cell populations are shown in **a**). 2D and 3D representations of an ES cell colony comprising two distinct populations of cells combinatorially expressing H2B–GFP; myr–mRFP1 or H2B–mCherry; GFP–GPI, respectively (**b–i**). White arrowheads depict: 1: GFP–GPI; 2: H2B–GFP; 3: H2B–mCherry and 4: myr–mRFP expression. Bright field image (**b**), green (**c**, **f**), red channels (**d**, **g**), merge of green and red channel (**e**, **h**) and overlay of bright field image onto green and red channel (**i**). Scale bar: 20 μm .