Published in final edited form as:

Methods Mol Biol. 2016 ; 1341: 101–110. doi:10.1007/7651_2015_214.

Expanding the utility of FUCCI reporters using FACS-based 'omics analysis

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Summary

The FUCCI indicator system is a powerful tool for spatio-temporal analysis of the cell cycle but its utility has been restricted so far to limited range of applications. Here we describe how to establish and validate the FUCCI system in murine pluripotent stem cells (PSCs) and describe the utility of transgenic FUCCI mice. We then describe how the FUCCI system can be used to generate material for a wide-range of 'omics-based applications in conjunction with FACS isolation of cells. This significantly broadens the potential applications of FUCCI reporters for studying the molecular basis of development and disease.

Keywords

FUCCI; cell cycle; stem cells; pluripotency; FACS

1. Introduction

The 'fluorescence ubiquitin cell cycle indicator' (FUCCI) system is a valuable tool for characterizing spatio-temporal regulation of cell division [1–5]. Sakaue-Sawano and colleagues genetically engineered two fusion proteins, monomeric Kusabira Orange with a

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Note 3.3.1. We established the FUCCI system in R1 murine ES cells. We seeded 0.5×10^6 cells per 10 cm dish, and then incubated for 3 days at 37°C with 5% CO₂ to obtain a confluent culture. Example tissues demonstrated here were isolated and cultured as previously described: tail tip fibroblasts [10], neonatal cardiomyocytes [11], fetal hepatocytes [12]. The number of cells, or tissues, needed to perform a given experiment will vary (see Table 1), and may require combining equivalent fractions from multiple sorts. Furthermore, single cell analysis of transcript expression is available [13] but currently uncommon.

Note 3.3.2. While trypsin (0.05%)-EDTA is often used to create single cell suspensions of mPSCs, we have found that Accutase is superior for reducing cell clumping that would otherwise disrupt the flow cytometer.

Note 3.3.4. Forward scatter and side scatter readings were collected in linear mode off the 488nm laser excitation. Usually two distinct populations are evident, where the cells appearing nearest to 0 on the X- and Y- axis are either dead cells or debris and the larger population represents viable cells.

Note 3.4.1. The amount of DyeCycle reagent to be added here may need to be optimized for specific cell lines. mPSCs have been known to efflux dyes and therefore an increased amount may need to be used if insufficient staining is observed.

Note 3.4.2. The incubation time with the dye can also be fine-tuned in order to optimize staining. Note 3.4.3. In this setup, forward scatter and side scatter were collected in linear mode off the 488nm laser excitation and used to exclude dead cells and debris. DyeCycle Violet fluorescence was excited by 405 nm laser excitation (Coherent) and detected using a 447/55 bandpass emission filter.

Note 3.4.4. The percentage of cells in cell cycle phases can be analyzed using the FlowJo Cell Cycle platform (<http://www.flowjo.com/>).

Cdt1 truncation mutant (mKO2-Cdt1) and monomeric Azami Green with a Geminin truncation mutant (mAG-Gem), which oscillate via post-translational control in order to indicate individual cell cycle phases with a unique fluorescent signature [4]. This system has proven to be a dramatic improvement compared to previous methodologies for interrogating cell cycle events in mammalian cells. Previously, the field relied heavily on pharmacological treatment to synchronize cells but this is problematic due to cytotoxicity and because cell cycle aberrations are frequently observed under these conditions.

Two recent reports have coupled the FUCCI indicator system to fluorescence-activated cell sorting (FACS) to expand the utility of this system to include molecular approaches such as qRT-PCR, western blots, chromatin immunoprecipitation (ChIP) [6,7] and at the 'omics levels, RNA-seq [6]. In these two cases, cultured pluripotent stem cells (PSCs) were used as a biological platform. In this report we describe in detail how to establish the FUCCI system in pluripotent stem cells (PSCs) and how to interpret the fluorescent signatures of cells. (Fig. 1). We then describe how to couple the FUCCI system with FACS isolation of cells in a distinct cell cycle phase from a heterogeneous population of PSCs and from tissues of transgenic mice (Fig. 2). Lastly, we demonstrate how implementation of this strategy followed by focused molecular analyses, such as transcript and protein expression analysis, and 'omics based analyses, such as proteomics, ChIP-seq, chromatin conformation capture assays (3C, 4C, 5C, HiC, ChIA-PET) [8] and epigenomics (DNA methylation) enhances the ability to characterize the molecular mechanisms involved in development and disease (Fig. 3).

2. Materials

The following materials are required for establishment, characterization, and FACS of FUCCI PSCs and primary cells.

1. FUCCI DNA Vectors (<http://www.mblintl.com/search/product-results.aspx?k=Fucci&submitsearch=%C2%A0&a=0&c=0&co=0&h=0&p=0&s=0&st=0&ct=0>)
2. FUCCI mice: <http://www.amalgaam.co.jp/products/advanced/fucci.html>
3. Mouse PSC culture medium: fetal calf serum (10%), KnockOut serum replacement (10%)(Life Technologies #10828-028), Leukemia Inhibitory Factor (1000 units/mL)(Millipore #ESG1107), Sodium Pyruvate (100 mM), L-glutamine (200 mM), Penicillin-Streptomycin solution (500 µg/mL), β-mercaptoethanol (0.1 mM). Combine these components into a base of Dulbecco's Modified Eagle Medium (DMEM) and run through a filter flask with 0.45 µm pore size.
4. Drugs for selection of FUCCI markers (Puromycin and Neomycin recommended for mPSCs)
5. Vybrant DyeCycle Violet (Life Technologies #V35003)
6. Dulbecco's Phosphate-Buffered Saline (DPBS; 1x)
7. 5 mL cell strainer tubes.

8. 5 mL round bottom collection tubes.
9. Accutase (Life Technologies #A1110501)
10. Anti- Kusabira Orange 2 (MBL International #M168–3)
11. Anti- monomeric Azami-Green 1 (MBL International #PM052)

3. Methods

3.1. Establishment of the Fucci system in PSC lines

Investigating potential connections between the cell cycle machinery and cell fate decisions has been a difficult task due to a lack of suitable tools. Previous studies relied on small molecules, such as aphidicolin and nocodazole, to synchronize cells in a particular cell-cycle phase, with the unintended complications of acute cytotoxicity, metabolic perturbations, and a diminished ability for cell cycle reentry [9]. Implementation of the Fucci system alleviates these complications and permits interrogation of cell cycle controlled mechanisms in PSCs and their derivatives.

1. Subclone the mKO2-Cdt1 DNA fragment into an appropriate expression plasmid vector (*see Note 1*)(Fig. 1A).
2. Transfect the mKO2-Cdt1 expression plasmid into cells.
3. Apply drug selection until un-transfected cells die off (*see Note .2*).
4. Establish a stable cell line by isolating and clonally expanding cells that display mKO2 fluorescence (*see Note .3*).
5. Subclone the mAG-Gem DNA fragment into an appropriate expression plasmid vector (Fig. 1A).
6. Transfect the mAG-Gem expression plasmid into cells from step 4.
7. Establish a stable cell line by isolating and clonally expanding cells that display mAG fluorescence (*see Note 4*).
8. Confirm that colonies contain cells expressing Fucci markers under a microscope (Fig. 3C, *see Note 3.1.5*)

¹-An appropriate plasmid expression vector should contain a promoter that drives constitutive expression of the DNA fragment; CAGi works well in R1 mPSCs (Fig. 3A). Also, each of the two Fucci reporters should be cloned into vectors containing drug selectable markers. Puromycin and neomycin selection are recommended using expression cassettes linking them to the fluorescent protein reporter component via an internal ribosome entry site (IRES). For R1 mPSCs, we recommend using puromycin at a concentration of 1 µg/mL and neomycin at a concentration of 200 µg/mL.

²-The time needed for drug selection to complete varies based on the selectable marker used. Puromycin usually kills untransfected cells within a few days and transfected cells will amplify after approximately 1 week. Neomycin can take slightly longer.

³-Single mKO2 positive cells can be isolated via FACS, or colonies can be picked under a fluorescent scope. It is unlikely that an entire colony will be mKO2 positive, as approximately 5–10% of mPSCs are in late G1 at any one time.

⁴-Cells successfully transfected with mAG-Gem can display a large range of mAG expression: G1 cells will not display mAG, S-phase cells will display low levels of mAG, and G2/M cells will display high levels of mAG.

Note 3.1.5.Expression of Fucci proteins can be further validated by immunofluorescent staining and/or western blot analysis using commercially available antibodies (*see Materials*).

3.2. Confirmation of FUCCI expression in mice

Transgenic FUCCI mice (*see Materials*) allow researchers to study aspects of cell cycle control as it relates to disease, tissue repair and regeneration, and aging. While maintaining colonies is straightforward, we have noted that they exhibit reduced fecundity in comparison to other wild type strains, as evidenced by small litter sizes. We have found it advantageous to outcross with a Swiss Webster mouse prior to experiments requiring large tissue numbers. In this situation, the following steps should be taken to screen for proper FUCCI marker expression in the offspring prior to experimentation.

1. Cut off a small (~1 mm) piece from the tail tip of a neonatal FUCCI mouse.
2. Place the section on a microscope slide in a drop of DPBS and add a coverslip.
3. Confirm positive mKO2 expression by observing under a fluorescence microscope using a green laser (488 nm).
4. Confirm positive mAG expression by observing under a fluorescence microscope using a red laser (561 nm).

3.3. FACS sorting of FUCCI cells

The FUCCI system has proven to be a valuable tool for characterizing spatio-temporal aspects of cell division. By combining FUCCI fluorescence with FACS isolation of cell-cycle fractions, it is now feasible to perform downstream analyses to better investigate hypotheses regarding molecular aspects of cell cycle control. Furthermore, using this highly reproducible protocol makes it possible to combine equivalent sorted fractions from multiple FACS isolations, allowing for large scale analyses, such as chromatin conformation capture assays (3C, 4C, 5C, HiC, ChIA-PET) [8] that require tens of millions of cells.

1. Determine an appropriate number of PSCs, or mice, required for intended analysis (*see Note .1*, Table 1, Table 2).
2. Create a single-cell suspension from mPSCs, or isolated tissues of interest (*see Note .2*).
3. Adjust the cell suspension concentration to 10,000,000 cells/mL.
4. Filter the cell suspension using cell strainer tubes appropriate for the FACS apparatus.
5. Initialize the flow cytometer by setting the 488 and 561 laser outputs to 100 mW and setting the voltage of the mKO2 detector to 406 and the mAG detector to 325 (*see Note 3.3.3*).
6. Run approximately 50,000 to 100,000 cells at a rate of 10,000 events per second to set gating.

Note 3.3.3. Parameters specified herein are for use with the Beckman Coulter MoFlo XDP cell sorter and will likely require customization for other sorters. In this setup, mKO2 fluorescence was excited by a 561nm laser and emission detected with a 586/30nm bandpass filter. mAG fluorescence was excited by a 488nm laser and emission detected with a 529/28nm bandpass filter. Both fluorescent protein emissions were measured in log and compensation was performed as needed to subtract overlapping emissions.

7. Gate out dead cells and debris by forward scatter versus side scatter gating (*see* Note .4).
8. Gate cells from step 8 by mKO2 versus mAG for the cell cycle fractions of interest (*see* Note 3.3.5)(Fig. 3C,D).
9. Sort cells into FACS collection tubes containing 200 μ L of culture medium, which will increase post FACS viability.

3.4. Validation of sorted fractions by DNA content

Different cell types will show distinct patterns of FUCCI marker expression, reflecting differences in rates of cell cycle progression (Fig. 2A). This can complicate FACS and downstream analysis. Therefore it is necessary to validate the sorted fractions using a DNA content stain and optimize the protocol for the cell type of interest.

1. Dilute FACS sorted cell cycle fractions and unsorted cells to a concentration of 10^6 cells/mL in 1x DPBS.
2. Aliquot 1 mL to a 5 mL polystyrene round-bottom FACS tube.
3. Add 1 μ L of Vybrant DyeCycle Violet to the tube (*see* Note .1).
4. Incubate for 30 minutes at 37°C in the dark (*see* Note .2).
5. Place on ice
6. Analyze DNA content of viable cells on a flow cytometer (*see* Fig. 2B & Note .3).
7. Plot cell count frequency versus DNA content to determine the purity of the FACS sort (*see* Fig. 2C & Note .4).

3.5. Conclusion

The FUCCI system has previously allowed researchers to characterize spatio-temporal aspects of cell division. The methods described here for establishing FUCCI PSC lines and primary cells from transgenic mice, coupled with the use of FACS, increases the capacity to perform a variety of 'omics type analyses that will reveal the cell cycle specific molecular mechanisms important for disease and development.

Acknowledgements

This work was supported by grants to SD from the National Institute of Child Health and Human Development (HD049647) and the National Institute for General Medical Sciences (GM75334). We thank Julie Nelson for assistance with FACS.

Note 3.3.5. Cells that are double negative (DN) for mKO2 and mAG are in the early G1 phase. Cells positive for mKO2 and negative for mAG (KO2) are in late G1 phase. Cells negative for mKO2 and with moderate mAG expression (AzL) are in S phase. Cells negative for mKO2 and highly positive for mAG (AzH) are in G2/M phase. Gating different percentages of each fraction will affect the purity and exact cell cycle phase composition of the sorted cells. The percentage of cells in each cell cycle phase can vary greatly amongst different cell types (*see* Table 2, Fig. 2A). For this reason, combining equivalent fractions from multiple sorts may be necessary for some downstream analyses.

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Fig. 1. The Fucci indicator system
(A) Diagram of Fucci reporters (B) Schematic linking fluorescent protein expression patterns with cell cycle phase. (C) Oscillations of Fucci reporter fluorescence during cell cycle progression.

Fig. 2. FACS isolation and validation of cell cycle fractions.

(A) Murine PSCs and primary cells from FUCCI mice can be isolated and used for imaging or isolation by FACS. Boxes on FACS profiles indicate gating; see Table 2 for the number of cells collected per gate. (B) FUCCI mPSCs stained with Violet Vybrant DyeCycle and analyzed for DNA content by flow cytometry. (C) Cell cycle fractions following FACS, stained with Violet Vybrant DyeCycle and reanalyzed for DNA content.

Fig. 3. Examples of downstream applications of FACS isolated cells.

Example analyses that can be performed following FACS. **(A)** 'Omics **(B)** Cell Size Analysis **(C)** Focused Analysis.

Table 1.

Example cell numbers needed for a variety of downstream analyses.

Experiment	Cell # needed
RNA-seq *	1 – 10 ⁵
qRT-PCR *	1 – 10 ⁵
Western Blot	5×10 ⁵
Proteomics	5×10 ⁶ - 10 ⁷
ChIP	10 ³ - 10 ⁶
3C, 4C, 5C, HiC, ChIA-PET	5×10 ⁷ - 1×10 ⁸

* Single cell analysis is available [13], but currently uncommon. Upper estimates are reflective of current standard technologies.

Table 2.

Approximate cell numbers from one confluent 10cm culture dish*, or 1 tissue!, and expected cell numbers for each cell cycle fraction following FACS from a starting population of 1 million cells.

Cell Type	Cell #	Early G1 (%)	Late G1 (%)	S (%)	G2/M (%)
mPSC*	2×10^7 *	2×10^6 (10%)	1×10^6 (5%)	2×10^6 (10%)	2×10^6 (10%)
Cardiomyocytes	2×10^6 !	2×10^5 (10%)	3×10^5 (15%)	1×10^5 (5%)	1×10^5 (5%)
Hepatocytes	2×10^6 !	2×10^5 (10%)	6×10^5 (30%)	1×10^5 (5%)	6×10^4 (3%)
Tail tip fibroblasts*	1×10^7 *	1.5×10^6 (15%)	6×10^6 (60%)	5×10^5 (5%)	5×10^5 (5%)