Methods in Molecular Biology (2016) 1341: 87–100
DOI 10.1007/7651_2015_255
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Published online: 11 July 2015

Imaging Pluripotency: Time-Lapse Analysis of Mouse Embryonic Stem Cells

Anna Pezzarossa, Ana M.V. Guedes, Domingos Henrique, and Elsa Abranches

Abstract

The current view of the pluripotent state is that of a transient, dynamic state, maintained by the balance between opposing cues. Understanding how this dynamic state is established in pluripotent cells and how it relates to gene expression is essential to obtain a more detailed description of the pluripotent state.

In this chapter, we describe how to study the dynamic expression of a core pluripotency gene regulator—Nanog—by exploiting single-cell time-lapse imaging of a reporter mESC line grown in different cell culture media. We further describe an automated image analysis method and discuss how to extract information from the generated quantitative time-course data.

Keywords: Stem cells, Nanog, Pluripotency, Heterogeneity, Dynamics, Time-lapse imaging

1 Introduction

Mouse Embryonic stem cells (mESCs) are pluripotent cells derived from the inner cell mass of the blastocyst. They are capable of both self-renewing and multilineage differentiation, enabling the regulation of cell number and type during early embryonic development (1, 2). Due to their properties, ESCs hold great potential for medical application (3, 4). However, to fully exploit this potential a conceptual understanding of the pluripotent state is required.

Pluripotency is controlled by a gene regulatory network (GRN) of transcription factors, with the triplet Oct4, Nanog, and Sox2 forming its core (5). They function together in activating other pluripotency genes, while repressing differentiation-promoting genes (6, 7). Oct4 and Sox2 can also drive the production of FGF4, a differentiation factor, functioning as lineage specifiers. In contrast, Nanog overexpression is able to suppress differentiation and maintain pluripotency even in the presence of FGF4

Electronic supplementary material The online version of this chapter (doi:10.1007/7651_2015_255) contains supplementary material, which is available to authorized users.

signaling. Thus, the current picture of the pluripotent state is that of a transient, dynamic state, maintained by the balance between opposing cues (8).

Contrasting with homogenous levels of OCT4 and SOX2, NANOG expression in mESCs is heterogeneous (9, 10), with a bimodal distribution between high- and low-expressing subpopulations. Possibly, these subpopulations have different responsiveness to differentiation stimuli (11, 12). This would allow keeping a pool of progenitors cells even in differentiating conditions.

NANOG levels fluctuate in individual cells; however, the source of this "noise" and its role remains unclear (13). One hypothesis is that fluctuations prime individual cells for differentiation, without marking definitive commitment. Different culture conditions (promoting self-renewal or differentiation) shift this equilibrium, without suppressing fluctuations. Understanding how this dynamic behavior is established in mESCs and how it relates to gene expression will lead to a more detailed description of the pluripotent state.

To investigate the dynamics of Nanog heterogeneity in mESCs, it is crucial to perform real-time monitoring of its expression in individual cells. This requires the use of a faithful reporter with good correlation between the temporal expression of reporter and endogenous proteins. Previously, we have established a NANOG reporter cell line (Nd) (10) containing a transgenic Bacterial Artificial Chromosome carrying a cDNA coding for a bright and fast degrading yellow fluorescent protein (VNP), inserted under the control of the Nanog regulatory regions. Our data show that the VNP reporter can be used as a faithful proxy for NANOG dynamic expression, and we have now improved this cell line to allow automated tracking, by introducing a constitutive mCherry reporter that is homogeneously expressed by all cells (NdC, from Nd plus mCherry).

In this chapter, we describe protocols to perform time-lapse fluorescence microscopy of single NdC cells expressing the Nanog fluorescent reporter (Section 3.2) under different self-renewing culture conditions (Section 3.1). We also describe a MATLAB-based software developed for automated image analysis (cell segmentation and tracking) (Section 3.3). These protocols provide a rapid and reliable way to follow in real-time fluorescent fluctuations of the reporter and extract information on the dynamic behavior of NANOG (Section 3.4), such as frequency and duration of expression, and amplitude of fluctuations.

2 Materials

Prepare all solutions under sterile conditions, in a laminar-flow cell culture hood (class II). mESCs should be preserved in cryogenic storage, with 10 % of DMSO as protective agent.

2.1 mESC Lines

The following mESC lines have been used:

1. E14tg2a cells, not genetically modified mESC line derived from 129/Ola mice blastocysts (a kind gift from Austin Smith's lab, University of Cambridge, UK).
2. NdC cells, a novel NANOG reporter mESC line derived from Nd mESCs (10) by stable integration of a mCherry-NLS reporter, which is expressed in all cells' nuclei.

2.2 Media and Reagents

All reagents stored at −20 °C should be removed from the freezer and leave to thaw overnight at 4 °C before use.

2.2.1 mESC Culture Media

1. Serum/LIF conditions:
   (a) Mix the following components in a 250-ml bottle:
   - 200 ml of sterile of 1× Glasgow Modified Eagle's medium (GMEM, GIBCO).
   - 2 ml of 200 mM glutamine (100×, GIBCO, stored in aliquots at −20 °C).
   - 2 ml of 100 mM Na pyruvate (100×, GIBCO, stored in aliquots at −20 °C).
   - 2 ml of 100× nonessential amino acids (GIBCO, stored at 4 °C).
   - 2 ml of 100× penicillin–streptomycin solution (GIBCO, stored in aliquots at −20 °C).
   - 200 μl of 0.1 M 2-mercaptoethanol (Sigma). The stock solution should be prepared in sterile ultrapure water, stored at 4 °C and used within 4 weeks).
   - 20 ml of fetal bovine serum (GIBCO ES-qualified FBS, inactivated 30 min at 55 °C and stored in aliquots at −20 °C).

   (b) Filter through a 0.2 μm filter unit into a new sterile flask, store at 4 °C and use within 1 month.

   (c) Supplement with 2 ng/ml Leukemia inhibitory factor (LIF) prior to use.
2. 2i/LIF conditions:
   (a) Supplement iStem medium (Stem Cells Inc.) (8) with respective supplement, store at 4 °C and use within 2 weeks. iStem media and respective supplement should be aliquoted, stored at −20 °C and always protected from light.

   (b) Supplement with 2 ng/ml LIF prior to use.

2.2.2 Poly-L-ornithine and Laminin Coating

1. Incubate plates with 1 μg/ml poly-L-ornithine/H_2O (Sigma) for 20 min at room temperature.
2. Wash twice with PBS.

3. Incubate with 10 μg/ml laminin (Sigma) in PBS for at least 3 h at 37 °C.
4. Aspirate laminin just before plating the cells.

2.2.3 Other Reagents

1. 0.1× Trypsin solution:
   (a) Prepare a 1× trypsin solution by mixing: 5 ml of 2.5 % trypsin (Gibco), 0.5 ml of heat-inactivated chicken serum, 0.1 ml of 0.5 M EDTA, and PBS to 50 ml.
   (b) Dilute the 1× trypsin solution with PBS.
2. 0.1 % Gelatine: dilute from 2 % in H_2O, tissue culture grade Gelatine solution (Sigma).
3. 2× Freezing medium: mix 800 μl of 1× Serum/LIF medium with 200 μl of DMSO Hybri-max (Sigma).

2.3 Equipment

2.3.1 Materials for Tissue Culture

1. Lab-Tek™ II Chamber Slide™ System (Nunc, cat.no. 155411).
2. Glass bottom MatTek culture plates (MatTek, cat.no. P35G-1.5-14-C).
3. 6-well multiwell tissue culture dish (Nunc, cat.no. 140675).
4. 60-mm tissue culture dishes (Nunc, cat.no. 150288).

2.3.2 Microscope

The microscope station for time-lapse imaging may vary. We used a 3i Marianas spinning disk confocal microscope (https://www.intelligent-imaging.com/marianas.php). Here is a list of the necessary components:

1. Inverted microscope.
2. High numerical aperture objectives (we used 63× oil immersion, a 40× is also recommended).
3. Laser sources. For multiple color acquisition at least two different wavelengths are necessary. For example, we used 488 nm and 561 nm, or 488 nm and 640 nm to ensure proper spectral separation.
4. Emission filter set adequate to the selected imaging wavelengths.
5. Spinning disk unit.
6. High resolution, high sensitivity CCD or EMCCD camera.
7. Incubator for temperature control and CO_2 supply.
8. CO_2 supply.
9. Motorized xy stage to acquire multiple positions.
10. Piezo for z-stacks acquisition.
11. Controlling computer and data acquisition software.
12. Computer for data analysis.
13. External storage for data files.

3 Methods

3.1 mESC Culture

All cell manipulations should be performed under sterile conditions, in a laminar-flow cell culture hood (class II). mESCs are grown at 37 °C in a 5 % (v/v) CO_2 incubator.

We routinely thaw and expand mESCs in Serum/LIF media, and only change to 2i/LIF culture media for specific experiments, but routine expansion can also be performed in 2i/LIF media.

3.1.1 Thawing and Routine Expansion of mESCs

1. Thaw mESCs in Serum/LIF medium:
   (a) Coat a 60-mm Nunc dish with 0.1 % Gelatine (for minimum of 10 min).
   (b) Heat 10 ml of Serum/LIF medium in a 37 °C waterbath.
   (c) Remove cells from cryogenic conditions and place in the 37 °C waterbath for approximately 1 min (medium color changes from yellowish to pink).
   (d) Resuspend cells in 4 ml of heated Serum/LIF medium and spin cells down (2 min, 165 × *g*).
   (e) Remove supernatant, resuspend cells in 5 ml of Serum/LIF medium and transfer to the previously gelatine-coated dish.
2. Change medium within 6–12 h.
3. Passage cells on the next day (*see* **Note 1**):
   (a) Wash cells twice with PBS.
   (b) Add 0.1× Trypsin (just enough trypsin solution to cover the cells) and place in the incubator for 2–3 min.
   (c) Knock the dish several times to dissociate cells and check under inverted microscope to ensure the cells have dissociated.
   (d) Add serum containing medium (Serum/LIF) to stop trypsinization, resuspend the cells by pipetting up and down and spin cells down (2 min, 165 × *g*).
   (e) Resuspend cells in Serum/LIF medium, count the viable cell number using trypan blue dye exclusion method and, when appropriate, take a sample for flow cytometry analysis (Section 3.1.3).
   (f) Inoculate cells at the desired cell density (for routine mESC expansion plate 3×10^4 cells/cm^2) in Serum/LIF medium, on freshly coated gelatine dishes.
4. Passage cells every other day (when cells reach 70–80 % confluence, *see* **Note 1**) at a constant plating density, in the desired cell culture media.

3.1.2 Plating mESCs for Time-Lapse Imaging

1. Two days before imaging, plate mESCs in Serum/LIF or 2i/LIF media as described in Section 3.1.1 (*see* **Note 2**). Plating of one well of a 6-well culture dish usually provides sufficient cells for the rest of the protocol.
2. One day before imaging, coat the required wells of a Lab-Tek Nunc chamber slide with poly-L-ornithine and laminin.
3. On the imaging day, dissociate mESCs (as described in Section 3.1.1) and count the viable cell number using trypan blue dye exclusion method.
4. Take a cell sample for flow cytometry analysis (Section 3.1.3) (*see* **Note 3**).
5. Inoculate cells at the desired cell density (2×10^4 cells/cm^2 or 3×10^4 cells/cm^2, respectively for Serum/LIF and 2i/LIF media, *see* **Note 4**) on poly-L-ornithing and laminin (*see* **Note 5**) coated Lab-Tek wells (approximately 500 μl of cell culture media in each well of a 8-well chamber slide) (MatTek dishes can also be used, *see* **Note 6**).
6. Incubate for 2–3 h (this is the time cells take to attach) before starting imaging.

3.1.3 Flow Cytometry Analysis

1. Resuspend $1–5 \times 10^5$ dissociated mESCs in 4 % (v/v) FBS in PBS.
2. Gate live cells based on forward scatter and side scatter.
3. Use E14tg2a mESCs as a negative control to define fluorescence gates and determine fluorescence in NdC ESCs (*see* Fig. 1 for typical flow cytometry profiles) (*see* **Note 3**).

3.2 Time-Lapse Imaging

1. At least 2 hours before the experiments turn on the microscope incubator, with temperature set at 37 °C.
2. Half an hour before the experiment, turn on the lasers and the CO_2 controller.
3. Place a drop of immersion fluid (water or oil) on the objective.
4. Position the sample in the microscope and focus.
5. Select one area without cells to be used as background reference, to account for day-to-day laser's intensity fluctuations (*see* **Note 7**).
6. Select position(s) to be imaged. Select area(s) with "healthy" cells. If using multiple positions, select starting from left up and moving to the right down. The cell density in the imaging area should be chosen according to the duration of the experiment (*see* **Note 8**). For long acquisitions (>24 h), sparsely populated areas are a better choice. Typically, we select 20 positions for >24 h movie.

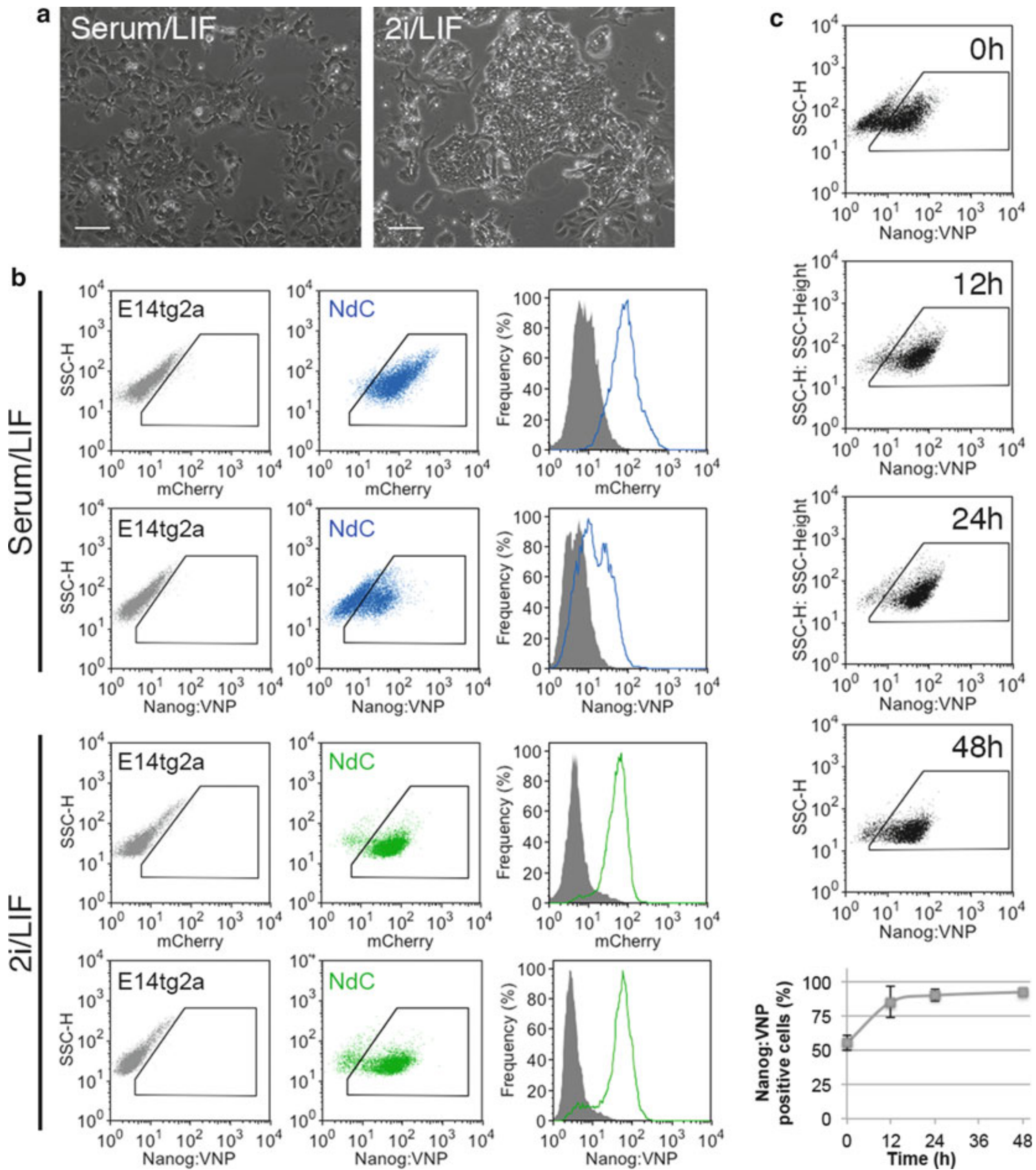

Fig. 1 Typical morphology and reporters flow cytometry profiles for NdC mESCs grown in Serum/LIF or 2i/LIF media. (**a**) Representative bright field images of NdC ESCs grown on poly-L-ornithine and laminin coated Lab-Tek wells. Cells were plated at a seeding density of 2 or 3 $\times$ 10^4 viable cells/cm^2, respectively for Serum/LIF or 2i/LIF media, grown at 37 °C incubator with a humidified atmosphere of 5 % CO_2 in air and images were taken after 36–48 h. Scale-bar: 100 μm. (**b**) Representative flow cytometry dot blots of Nanog:VNP and mCherry for E14tg2a (negative control, denoted in *gray*) and NdC cells (denoted in *blue* or *green*, respectively for Serum/LIF or 2i/LIF conditions). Positive gate regions were designed based on negative controls profiles. (**c**) Time course of VNP-positive cells, determined by flow cytometry, following transfer of NdC cells from serum/LIF to 2i/LIF medium

7. To allow for multiple positions measurement, record each position in the acquisition software.
8. If acquiring a z-stack set the lower and higher plane and the distance between planes (*see* **Note 9**).
9. Set the acquisition parameters: exposure time, time binning, and channels to be acquired. Contemporary acquisition of a phase contrast image is recommended.
10. Set imaging interval. Typical interval for short movies is around 2 min, while for longer movies images are acquired every 10/15 min.
11. Run a test acquisition.
12. Set the total experiment duration and begin acquisition.
13. It is advised to monitor the first half hour of acquisition to ensure that everything is running smoothly and, for long acquisitions, to regularly check on the status of the experiments.
14. At the end of the acquisition, before beginning data analysis, review the movies to check the quality of the acquisition.
15. Export movies. The final format will depend on the platform. In our case, each image in each channel was saved as a separate .tiff file, whose name contained information about the channel and the sequence. Different imaging platform might have different export options.

3.3 Tracking

Here we describe the procedure used in our lab to segment and track cells, based on a MATLAB software we have developed. The software is available upon request. It is also possible to perform the general steps of the process using different imaging software (e.g., ImageJ, Imaris).

1. Background subtraction. The software creates a mean image of the area without cells in each channel. This image is subtracted from all the subsequent images in each channel.
2. The software recreates a time series movie for each channel starting from the single .tiff images. (For other platform this step might not be necessary, as the files might be already exported as sequences.)
3. Segmentation—critical step. The software identifies each cell in a frame. Generally, it applies a threshold and morphological reconstruction to separate the fluorescent nucleus from the background. After segmentation, objects whose area is too big or too small to be a nucleus are removed. Segmentation is performed on the sequence of mCherry nuclear marker (Fig. 2a, c, d).
4. Inspect segmentation outcome and manually correct for mistakes.

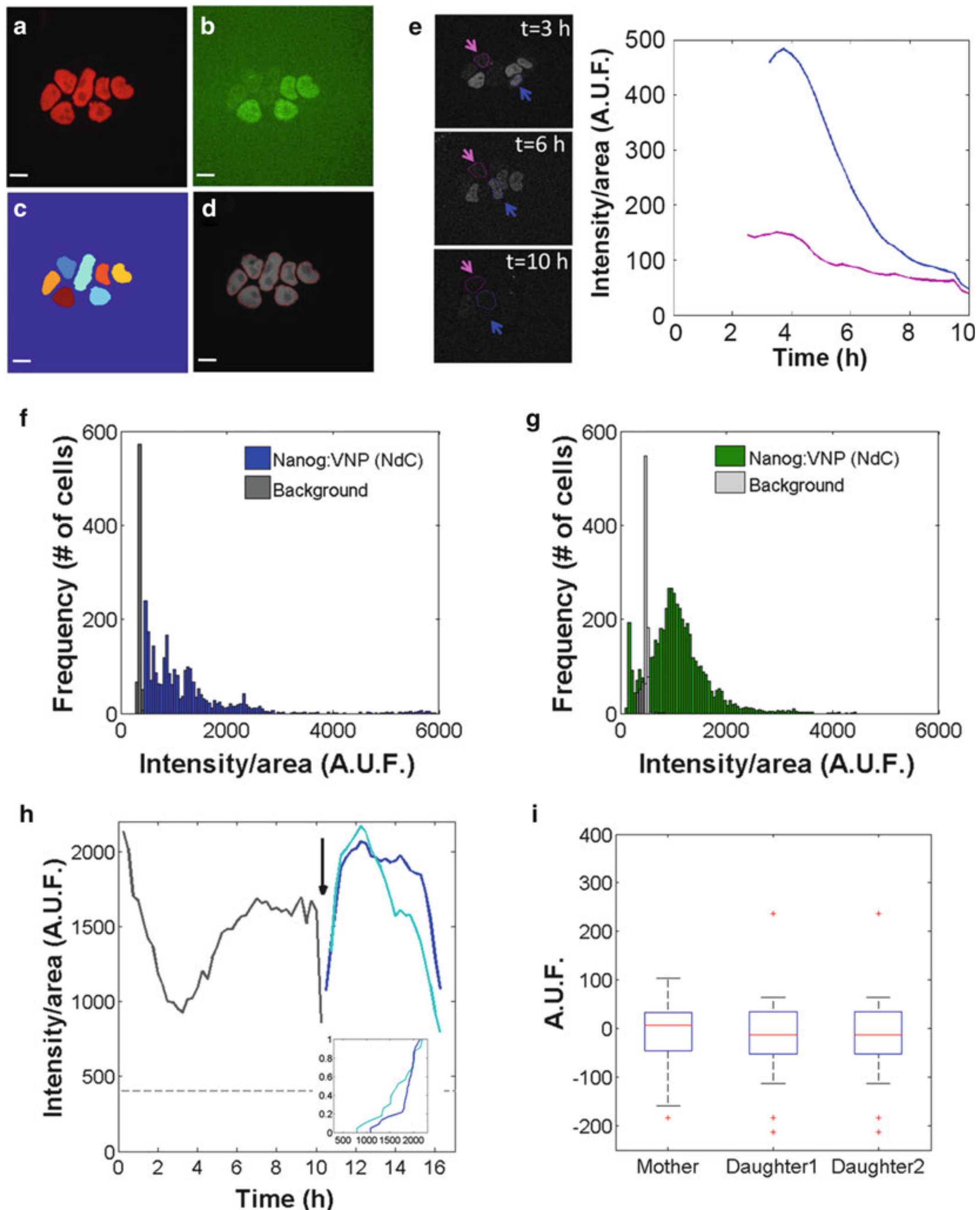

Fig. 2 Tracking of NdC mESCs and data analysis. (**a**) Representative mCherry nuclear stain of NdC cells grown in Serum/LIF. The image was colorized with ImageJ to aid visual recognition. (**b**) Representative Nanog:VNP nuclear stain of NdC cells shown in (**a**). The image was colorized with ImageJ to aid visual recognition. (**c**) Segmentation output, based on image in (**a**). Each cell is correctly identified and labeled according to a color code. (**d**) Nuclear perimeter of NdC cells, determined from (**b**) and overlaid to the original image (**a**). (**e**) Nanog:VNP fluorescence time trace of two NdC cells. (**f**) Histograms for Nanog:VNP fluorescence levels for NdC (*blue*) and E14tg2a (*gray*, negative control) cells grown in Serum/LIF media. All collected time points were

5. Labeling and tracking. Each cell is assigned a unique numerical label. Subsequently, tracking connects cells from consecutive images. To track cells between two frames, the overlap between two consequent frames is calculated. The process proceeds backwards, from the last frame to the first; thus, each cell division results in the disappearance of one cell.
6. Manually correct tracking. Each cell is represented with a color-coded nuclear contour (Movie 1, see Extras.springer.com) (*see* **Note 10**).

3.4 Data Analysis

A series of different parameters can be extracted from the movies. Here, we report a list of the most commonly used.

3.4.1 Fluorescence Intensity

1. Fluorescence distributions. The fluorescence intensity is calculated for each cell in each image and calculated values can be plotted as a histogram. This should be done for each culture media tested and background levels should be determined using ESCs with no fluorescent reporter (Fig. 2f, g, respectively for serum/LIF and 2i/LIF conditions) (*see* **Note 11**). To account for cell size discrepancies, the fluorescence intensity is usually normalized by the cell area. In the frames immediately before and after cell division, the cell spread out and fluorescence is lost. Therefore, these frames are usually discarded.
2. Fluorescence variation over time. Plot the fluorescence intensity calculated for each cell in each image vs. time. The intensity traces are retrieved for both channels (mCherry and VNP) (Fig. 2a, b). In Fig. 2e, the tracking of the VNP levels of two cells is illustrated.
3. Smoothing traces. When imaging with large time interval between frames (>5 min), it can be useful to apply a moving average smoothing to the intensity trace. Usually, three frames smoothing provides good results. The smoothing procedures allow to remove variation in intensity due to technical deviations (e.g., changes in focus or laser intensity).
4. Fluctuation Index. To compare the dynamic range of fluctuations in different conditions, the amplitude between the maximum and minimum fluorescence levels detected along an interphase can be calculated for each cell (Table 1).

Fig. 2 (continued) used. (**g**) Same as (**e**) for NdC (*green*) and E14tg2a (*gray*, negative control) cells grown in 2i/LIF media. (**h**) Nanog:VNP fluorescence time trace of a mother NdC cell and respective progeny, grown in Serum/LIF. *Arrow* indicates division time. *Inset plots* show the empirical cumulative distribution functions for each sister cell. (**i**) *Boxplot* representing the rates of gain and loss of fluorescence for the tracks shown in (**h**). Scale-bar for (**a**–**d**): 10 μm

Table 1
Cell cycle and fluctuation index parameters extracted from single cell time-lapse analysis of a Nanog reporter mESC line grown in two different cell culture media (Serum/LIF or 2i/LIF). Parameters relaying on fluorescence values (fluctuation index and rates) may vary depending on the microscope system used for cell tracking

Parameter	Serum/LIF	2i/LIF	References
Cell cycle (hours)	10 ± 2.3 ~12.6	12.3 ± 2.2 ~12.7	This chapter [12]
Fluctuation Index (A.U.F.)	486 ± 410 459 ± 448*	1033 ± 730 560 ± 311*	This chapter [12]

Note: these values were calculated considering only fluctuating cells

3.4.2 Cell Cycle Length

This is calculated as the time between two subsequent cell divisions (Table 1). Only cells for which both initial and final mitosis are observed should be considered.

3.4.3 Rate of Loss or Gain of Fluorescence

These parameters are estimated from the fluorescence vs. time tracks. To calculate the paces at which fluorescence changes occur, the rates of fluorescence increase and decrease (in arbitrary fluorescence units per time) for all cells are calculated from which mean values may be estimated. This data can also be plotted in the form of a histogram, where values around zero denote cells that show no fluctuations, and positive (gain rates) or negative (loss rates) values denote fluctuating cells (*see* **Note 12**).

3.4.4 Sister Cells Analysis

Using fluorescence vs. time tracks, the kinetics of sister cells can be analyzed (Fig. 2h). Different statistical tests can be used, such as the empirical cumulative distribution function (ecdf) and the Kolmogorov–Smirnov (K–S) test, to evaluate whether the curves belonging to sister cells come from the same distribution.

4 Notes

1. Always monitor cell morphology under an inverted microscope (typical cell morphologies are shown in Fig. 1) and passage cells when 70–80 % confluence is reached. If cells have not reached this after 48 h, keep changing the media every other day until the required confluency is achieved.
2. Upon culture media change, adaptation of mESCs is fast, and changes in NANOG expression are already detected after 12 h, stabilizing after 24 h (*see* Fig. 1c). These changes are accompanied by morphological alterations with the use of 2i/LIF media, resulting in more tightly packed mESCs colonies and a

reduction in flattened differentiated cells (10). Consequently, cell attachment in 2i/LIF conditions is poorer (especially on gelatin-coated dishes), and care should be taken not to wash away cell clusters during the passaging procedure.

3. When working with reporter cell lines, a routine check of the reporter expression is desirable. This can be quickly and quantitatively performed by flow cytometry analysis, from which the histogram of expression levels can be extracted.
4. The plating density may need to be optimized for each individual cell line, and for each cell culture media used. The chosen density should ensure that the lag phase of cell growth is minimized, and that confluency is only reached after 48 h. Typical plating densities for mESCs: $1–4 \times 10^4$ cells/cm^2. For longer time-lapse movies (more than 48 h), media change is required to ensure healthy cell maintenance.
5. mESCs are routinely grown on gelatin-coated polystyrene plates but for imaging purposes glass bottom plates have to be used. In these conditions, attachment is usually poorer and mESCs tend to grow even in more tightly compact colonies, hindering automated tracking of cells. The use of more efficient attachment substrates is therefore required and coating with poly-L-ornithing and laminin is a good alternative (fibronectin can also be used). Coatings should always be freshly prepared.
6. When Mattek dishes are used, resuspend cells in 400 μl media, add the mixture to the glass-covered portion of the dish, and incubate at 37 °C. After 2–3 h add 2 ml of preheated media at 37 °C. Incubate for 1–2 h at 37 °C and proceed to imaging. Equilibration times at 37 °C are critical to avoid cell detachment upon transfer for the microscope settings.
7. Laser intensity and illumination time should be previously determined, since they depend on the chosen fluorophore and the duration of the experiment. Ideally, these parameters should be optimized to minimize photobleaching and cell death, while allowing for high image quality. We suggest running a series of test experiments to see which values fit best the cell sample. Once the parameters have been chosen, they should be kept the same for all the experiments to allow for comparison.
8. When planning to monitor for longer time periods, it is better to choose areas with lower cell density for imaging. If cells grow in layers it becomes difficult or not possible at all to track them automatically or even manually.
9. When performing time-lapse movies, it is recommended to acquire a z-stack, by setting the lower and higher plane and the distance between planes. This will prevent loss of cells due to cell movement across planes, which is likely to occur during cell division.

10. The software offers the opportunity to choose between removing or keeping cells on the border of the image. Removing cells on the border reduces the number of incomplete tracks (a cell that goes in and out of the imaging area will be counted as a new one at each reappearance).
11. Different media result in different fluorescent backgrounds. Thus, for every media used, it is necessary to acquire a set of images of nonfluorescent cells (at each chosen wavelength) to determine the background level of fluorescence. This is necessary especially when comparing results from different conditions. On top of this control, day-to-day variation in laser intensity might cause slight changes in background fluorescence. Thus, it is important to acquire a movie of an area without cells to use as a background reference.
12. Fluctuation rates are calculated as relative values and are independent of the absolute number of reporter molecules per cell. Consequently, two individual mESCs can show similar fluctuation rates while having different reporter protein concentrations.

Acknowledgments

This work was supported by Fundação para a Ciência e Tecnologia, Portugal [SFRH/ BPD/78313/2011 to E.A., SFRH/BD/ 80191/2011 to A.M.V.G. and PTDC/SAUOBD/100664/ 2008].

Electronic Supplementary Material

Below is the link to the electronic supplementary material.Movie 1Representative time-lapse movie where each cell is represented with a color-coded nuclear contour (MOV 872 kb)

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