

Chapter 2

Live-Cell Imaging Combined with Immunofluorescence, RNA, or DNA FISH to Study the Nuclear Dynamics and Expression of the X-Inactivation Center

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Abstract

Differentiation of embryonic stem cells is accompanied by changes of gene expression and chromatin and chromosome dynamics. One of the most impressive examples for these changes is inactivation of one of the two X chromosomes occurring upon differentiation of mouse female embryonic stem cells. With a few exceptions, these events have been mainly studied in fixed cells. In order to better understand the dynamics, kinetics, and order of events during differentiation, one needs to employ live-cell imaging techniques. Here, we describe a combination of live-cell imaging with techniques that can be used in fixed cells (e.g., RNA FISH) to correlate locus dynamics or subnuclear localization with, e.g., gene expression. To study locus dynamics in female ES cells, we generated cell lines containing TetO arrays in the X-inactivation center, the locus on the X chromosome regulating X-inactivation, which can be visualized upon expression of TetR fused to fluorescent proteins. We will use this system to elaborate on how to generate ES cell lines for live-cell imaging of locus dynamics, how to culture ES cells prior to live-cell imaging, and to describe typical live-cell imaging conditions for ES cells using different microscopes. Furthermore, we will explain how RNA, DNA FISH, or immunofluorescence can be applied following live-cell imaging to correlate gene expression with locus dynamics.

Key words Live-cell imaging, X chromosome inactivation, Fluorescent in situ hybridization (FISH), Embryonic stem cells, Tet operator, Tet repressor

1 Introduction

Differentiation of embryonic stem cells (ESCs) is accompanied by changes in chromatin structure and content. Live-cell imaging of chromosomes and chromatin is a very powerful means of studying the dynamics, kinetics, and order of events that occur during ESC differentiation, at the single-cell level. One of the hallmarks of differentiation in female ESCs is random X chromosome inactivation (XCI), the process ensuring dosage compensation in female mammals, whereby either the maternal or paternal X chromosome becomes transcriptionally silenced during the first few days of ESC

differentiation. Random XCI is regulated by an X-linked region known as the X-inactivation center (Xic) [1] which contains the genes encoding for the long noncoding RNA *Xist* [2–4] and its antisense transcript *Tsix* [5] as well as numerous long-range regulatory elements [6]. Monoallelic upregulation of *Xist* is the first step during random XCI. It is regulated by multiple dynamic events occurring as soon as ES cell differentiation begins. The upregulation of *Xist* from only one of the two X chromosomes is partly ensured by the monoallelic downregulation of its antisense repressor, *Tsix*. The transient downregulation of *Tsix* on one allele has been linked to transient homologous associations between the two Xics at this locus during early ES cell differentiation. Xic trans-interactions or “pairing” events have been proposed to be involved in both sensing the number of X chromosomes that are present [7] and establishing asymmetric *Tsix* expression, such that monoallelic *Tsix* downregulation leads to monoallelic *Xist* upregulation [7–10].

Dissecting the order and function of these dynamic and transient events represents a perfect illustration of the need for live-cell imaging techniques in ESCs and their differentiated derivatives. Several questions including the duration of the pairing process, as well as the extent to which close proximity is actually followed by complete overlap between the two Xic loci, can be addressed using live-cell imaging of Xic dynamics during ESC differentiation. Live-cell imaging has also enabled Xic pairing events to be linked with a functional outcome. When combined with subsequent RNA FISH, it was found that pairing of the two Xics is followed by transient, asymmetric downregulation of *Tsix* [10]. Based on this combination of live and fixed cell analyses, it has been concluded that Xic pairing is likely to be an important upstream event for symmetry-breaking between the two Xics.

In order to visualize Xic dynamics in living ESCs, we have exploited the bacterial Tet operator (TetO)/Tet repressor (TetR) system. The TetO array can be visualized in living cells by transient or stable expression of TetR fused to fluorescent proteins (e.g., mCherry) which can bind with high affinity to the TetO array. In our study, a multicopy array of 224 single TetO sites was inserted by homologous recombination into a region adjacent to *Tsix* within the Xic in female mESCs (Fig. 1). An ES cell line in which both X chromosomes were tagged in this way was then derived [10] and a TetR-mCherry transgene was used to follow the dynamics of the Xic in living cells, using a DeltaVision microscope. This was the first report of live-cell imaging of an endogenous locus in ESCs and their early differentiated derivatives [10].

In this chapter we present a detailed description of the current methodology that we use to investigate nuclear and chromosome dynamics in ES cells, using the previously described female ESC line which carries two TetO-/TetR-tagged Xic loci. Several aspects

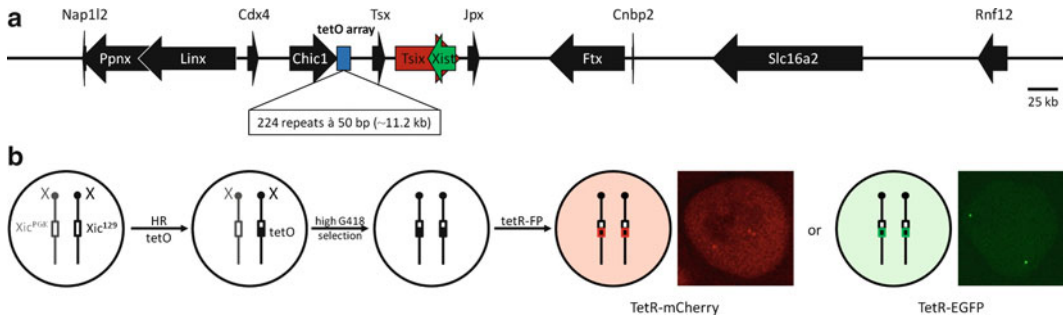


Fig. 1 (a) Schematic representation of the X-inactivation center (Xic), adapted from ref. 10. A TetO array containing 224 repeats was integrated by homologous recombination close to the *Chic1* gene in the X-inactivation center approximately 40 kb upstream of the 5' end of *Tsix*. (b) Integration of the TetO array resulted in a heterozygous cell line which has been treated with high concentrations of G418 to generate ES cell lines homozygous for the integrated TetO array [10]. TetR-FP binds the TetO arrays in the Xic and can be detected by live-cell imaging

will be covered, including (a) rapid acquisition live-cell imaging at high resolution in time, to study the dynamics and kinetics of fast processes; (b) imaging over more extended periods with less frequent acquisitions, to assess events over entire cell cycles and during differentiation; and (c) combination of live-cell imaging and fixed cell analysis (e.g., RNA FISH, immunofluorescence), for example, to follow Xic pairing dynamics and correlate this with *Tsix/Xist* expression patterns using RNA FISH [10]. We also mention recent advances that we have made concerning longer-term live-cell imaging of ESC using the OMX microscope which provides increased sensitivity and decreased phototoxicity for live-cell imaging.

2 Materials

2.1 Tissue Culture

1. ESC medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15 % fetal calf serum (batch tested FCS for ES cells), 0.2 % (v/v) 2-mercaptoethanol, and 1,000 U/ml leukemia inhibitory factor (LIF).
2. Differentiation medium: DMEM supplemented with 10 % FCS, 0.2 % (v/v) 2-mercaptoethanol, and 100 nM all-*trans*-retinoic acid (RA).
3. Freezing medium: 10 % dimethyl sulfoxide (DMSO) in FCS.
4. 6, 24, 96-well tissue culture plates.
5. Tissue culture flasks.
6. 10-cm tissue culture dishes.
7. 10× phosphate-buffered saline (PBS).

8. Double-processed tissue culture water.
9. 0.1 % gelatin in 1× PBS.
10. Alternative pre-coat for live-cell imaging: poly-d-lysine or human plasma fibronectin-purified protein.
11. 0.05 % trypsin-EDTA (1×) with phenol red.
12. 50 mg/ml hygromycin B.
13. Lipofectamine 2000 transfection reagent.

2.2 Live-Cell Imaging

1. 12, 18, and 30 mm #1.5 glass round coverslips.
2. MatriPlate 96-well glass-bottom microwell plate (MatriCal Bioscience).
3. Ludin chamber type 3 (Life Imaging Services).
4. Non-coat 35-mm petri dish with #1S 12 mm coverslip with grid (Matsunami Glass Ind.).
5. Non-coat 35-mm petri dish with #1.5 glass coverslip (MatTek Corp).
6. Fine forceps.
7. Paraffin.
8. DeltaVision Core (Applied Precision) equipped with a CoolSNAP HQ2 camera and an incubator chamber with CO₂ perfusion system (Okolab).
9. DeltaVision OMX V3 (Applied Precision) equipped with three Evolve back illuminated EMCCD cameras (Photometrics), three laser lines (405, 491, and 561 nm, powered with 200 mV, respectively). Speckles are removed by shaking of the multimode fiber at 4 kHz; OMX is equipped with an objective heater system (Applied Precision) and a heating stage (Applied Precision). Glass-bottom dishes with a 0.17 mm thickness (MatTek) are placed in a cell observation room (adapted from Applied Precision Instrument) and placed on a heating sample holder (custom made). The observation room includes a heating lid (adapted from Bioprotechs) and a temperature probe (adapted from Bioprotechs) which can be placed directly in the medium to monitor the temperature in the culture dish. CO₂ is perfused through the heating lid using a LIS CO₂ controller system.

2.3 Immunofluorescence/RNA/DNA FISH

1. Fixation solution: 3 % paraformaldehyde in PBS (freshly prepared, pH adjusted).
2. Permeabilization solution: 0.5 % Triton X-100 in PBS supplemented with 2-mM vanadyl ribonucleoside complex (VRC).

3. Antibody dilution solution: 1 % bovine serum albumin (BSA) in 1× PBS supplemented with 500 U/ml RNase inhibitor (e.g., RiboLock 40 U/μl).
4. 70 % Ethanol (v/v).
5. Nick translation kit (Abbott) containing nick translation enzyme, dNTP solutions, and 10× nick translation buffer.
6. Spectrum green dUTP (Abbott).
7. Spectrum red dUTP (Abbott).
8. Cy-5 dUTP.
9. Formamide (FA). Upon opening, aliquot immediately and keep at −20 °C.
10. Mouse Cot-1 DNA (Invitrogen).
11. DNA, molecular biology grade from fish sperm (Roche).
12. 3 M sodium acetate pH 5.2.
13. 10 mg/ml RNase A.
14. 5,000 U/ml RNase H.
15. 20× SSC buffer concentrate.
16. 2× Hybridization buffer: 40 % (w/v) sodium dextran sulfate, 20× BSA, 400-mM VRC in 4× SSC.
17. Wash buffer: 50 % formamide/2× SSC pH 7.2–7.4.
18. DNA counterstaining solution: 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) in 2× SSC.
19. Mounting solution: 90 % (v/v) glycerol, 0.1 % (w/v) *p-phenylenediamine*, pH 9.0 in PBS.
20. Eppendorf Centrifuge 5417R.
21. Eppendorf Concentrator plus.
22. Eppendorf Thermomixer comfort.
23. Liquiport Liquid pump.
24. Shake'N'Bake Hybridization Oven.

2.4 Plasmids

1. pBROAD3-TetR-ICP22-mCherry.
2. pBROAD3-TetR-ICP22-EGFP.
3. pBROAD3-mCherry-PCNA.

2.5 ES Cell Lines

1. PGK 31'-60 (PGK 2TetO TetR-mCherry).
2. PGK 31'-T12 TetR-ICP22-EGFP 12C (PGK 2TetO TetR-EGFP).

3 Methods

3.1 General Tissue Culture

All of the protocols described here apply to ESCs that grow in feeder-free conditions. Prior to live-cell imaging, ESCs or their differentiated derivatives should be growing optimally (good colony morphology, minimal cell death). Suboptimal cells should never be used for live-cell imaging, as they will rapidly die or show low/aberrant fluorescence. Usually it can take several days, and sometimes more than one passage for ESCs, to reach optimal conditions. Furthermore, imaging should be performed a minimum of 24-h post-plating of the cells onto the coverslip/in the chamber.

3.1.1 Thawing and Culturing ESCs

1. Thaw cells from liquid nitrogen in a water bath at 37 °C and resuspend them in an appropriate amount of ESC medium.
2. Pellet cells for 5 min at 180×*g* at room temperature in a 15-ml Falcon tube.
3. Resuspend the cell pellet in an appropriate amount of medium and distribute to a well or flask pre-coated with 0.1 % gelatin. Cells are grown as an adherent culture in wells or flasks and are split every 2–3 days in ESC medium.

3.1.2 Passaging ESCs and Plating for Imaging

1. Wash 70–80 % confluent cells once with pre-warmed 1× PBS (37 °C).
2. Incubate the cells for 8–10 min at 37 °C in 0.05 % trypsin-EDTA.
3. Stop trypsinization by addition of ESC medium and resuspend cells by vigorous pipetting with a 5-ml pipette (foaming indicates optimal dissociation). Split cells 1:5 to 1:10 (depending on the ESC line) into gelatin-coated wells or flasks. For live-cell imaging, count and distribute appropriate cell numbers onto pre-prepared, gelatin-coated petri dishes or coverslips.

3.1.3 Freezing Cells

1. Centrifuge cells after trypsinization and resuspension at room temperature at 180×*g* for 5 min.
2. Aspirate medium and resuspend cells in freezing medium and distribute to freezing tubes.
3. Freeze cells gradually at –80 °C, then transfer to liquid nitrogen the next day for long-term storage.

3.2 Generation of Stably Transgenic ESC Lines for Live-Cell Imaging Purposes

Imaging of chromosome dynamics can be performed by transient expression of TetR-FP in ES cell lines containing TetO arrays. However, it should be noted that optimal imaging results can only be obtained using ESC lines stably expressing TetR-FP. The following section describes in brief the general procedure for the

derivation of ESC lines stably expressing a TetR-FP fusion protein. The example given involves co-transfection of plasmids expressing the TetR-FP fusion proteins, as well as a plasmid expressing a selectable marker.

3.2.1 Transfection

1. Grow ESCs to 70–80 % confluence in a well of a 6-well plate.
2. Change ES medium 2 h prior to transfection.
3. Perform transfection with Lipofectamine 2000, following the manufacturer's instructions, using 3 μg of the TetR-FP expression vector (e.g., pBROAD3-TetR-ICP22-mCherry [10]) mixed with 0.2 μg selection marker expression plasmid (e.g., hygromycin resistance) per transfected well.
4. Incubate overnight.
5. Change medium and trypsinize the transfected cells the following morning and plate out 10 % and 90 % of the cells onto two pre-gelatinized 10-cm tissue culture dishes and culture the cells in ES medium for another day.
6. On the following day, change ES medium and start selection by adding the appropriate drug (e.g., hygromycin B; *see* **Note 1**).
7. After selection begins, medium should be changed every day until clone picking. Nonresistant cells should die during the next 1–5 days depending on the drug used.

3.2.2 ESC Clone Picking

1. Discrete ESC colonies should become visible by eye after approximately 7–10 days of culture. Colonies should be picked when they are still smaller than 1 mm and should not be allowed to grow too large or become necrotic in the center as this will increase the chances of differentiation and loss.
2. Wash the dish once with 10 ml 1 $\times$ PBS. Fill the dish with 5 ml PBS.
3. Pick single colonies in 30 μl PBS using a 200- μl pipette tip and pipette them into individual wells of a 96-well plate.
4. Add 30 μl of 0.05 % trypsin-EDTA per well to the picked colonies and incubate at 37 °C for 8 min.
5. Add 150- μl ES medium to stop trypsinization and dissociate the cells of the colony into a single-cell suspension by pipetting using a multi-pipette and 200- μl tips.
6. Distribute the dissociated cells to a pre-gelatinized 96-well plate. Change medium daily until cells grow to a confluence of 60–70 %.

3.2.3 Screening of Drug-Resistant Clones for the Presence and Expression of the TetR-FP Transgene

1. Split cells 1:3 into a pre-gelatinized 96-well plate (for future propagation) and a pre-gelatinized 96-well plate with glass-bottom dish (for screening).

Table 1
Typical numbers of ESCs seeded at day –1 for live-cell imaging during ESC differentiation with LIF withdrawal and addition retinoic acid in PGK derived cell lines

Day of differentiation	Number of seeded cells
Day 0	$\geq 5 \times 10^4 \text{ cm}^{-2}$
Day 0.5	$\sim 4 \times 10^4 \text{ cm}^{-2}$
Day 1	$\sim 3 \times 10^4 \text{ cm}^{-2}$
Day 1.5	$\sim 2 \times 10^4 \text{ cm}^{-2}$
Day 2	$\sim 1.75 \times 10^4 \text{ cm}^{-2}$
Day 2.5	$\sim 1.5 \times 10^4 \text{ cm}^{-2}$
Day 3	$\sim 1.25 \times 10^4 \text{ cm}^{-2}$
Day 3.5	$\sim 1 \times 10^4 \text{ cm}^{-2}$
Day 4	$\leq 1 \times 10^4 \text{ cm}^{-2}$

2. The clones can be screened in the 96-well plate with glass bottom using the DeltaVision microscope to detect expression of TetR-FP and visibility of TetO loci (*see* **Note 2**).

3.2.4 Propagation of Selected Clones

1. When clones have reached 70–80 %, confluence in the remaining 96-well plate transfers them into single, pre-gelatinized wells of a 48-well or 24-well plate by trypsinization. Each clone is likely to have its own growth kinetics and should only be passaged when it appears optimal in terms of confluence and morphology.
2. Repeat the procedure to transfer clones from the 24-well plate to 6-well plates. Cells can be frozen down and stored at this stage (6-well)-or further propagated in 25-cm² flasks after culturing them in 6-well plates.

3.3 Live-Cell Imaging of ESCs

3.3.1 Preparation of ESCs for Imaging Using a Ludin Chamber

1. Sterilize 12-mm coverslips (*see* **Note 3**) by flaming with ethanol and distribute sterile coverslips in the wells of a 6-well tissue culture plate (three coverslips per well). Pre-coat coverslips with 0.1 % gelatin in PBS (*see* **Note 4**). Aspirate the pre-coating solution before distribution of the cells.
2. Seed cells at day –1 according to a scheme that provides sub-confluent cell density (*see* **Note 5**) on the day of the imaging experiment. Typical cell densities at day –1 are depicted in Table 1 (as mentioned above, ESCs should be in optimal condition for live-cell imaging).

3. If differentiating cells are required, start differentiation (day 0) by washing out ESC medium and adding differentiation medium. Between the medium changes, wash the cells three times with pre-warmed 1× PBS to fully remove any traces of ESC medium.
4. Change medium 1–2 h before imaging (*see* **Note 6**).
5. Mount the coverslip into the Ludin live-cell chamber (*see* **Notes 7 and 8**): Take the coverslips from the 6-well plate with a pair of fine forceps and place it into the bottom of the Ludin chamber with cells facing up. Place a 12 mm rubber ring on top of the coverslip. Carefully screw the top parts of the Ludin chamber on the bottom part. Fill the Ludin chamber with 0.5–1-ml pre-warmed ESC medium or differentiation medium. Place a 30-mm glass coverslip on top of the chamber to prevent evaporation.
6. Place the Ludin chamber on the objective table of the DeltaVision microscope after putting immersion oil on the objective. Place the CO₂ perfusion system on the objective table (*see* **Note 9**). Focus the cells in the bright-field mode using the eyepiece.
7. Start imaging (*see* Subheading **3.3.3**).

3.3.2 Preparation for Imaging Using Plain or Gridded 35-mm Petri Dishes (See Note 10)

1. Pre-coat the surface of the petri dish with 0.1 % gelatin in PBS (*see* **Note 4**).
2. Follow **steps 2–4** of Subheading **3.3.1**.
3. Place the petri dish on the objective table of the DeltaVision microscope after putting immersion oil to the objective.
4. Note the orientation of the petri dish on the objective table (*see* **Note 11**).
5. Place the CO₂ perfusion system on the objective table (*see* **Note 8**). Focus the cells in the bright-field mode using the eyepiece.
6. Note the exact position of the imaged cells and colonies on the gridded coverslip in the petri dish using the bright-field mode and the numbered grid on the coverslip (also *see* Subheading **3.4**) and memorize these positions using the microscope software.
7. Start imaging (*see* Subheading **3.3.3**).

3.3.3 Live-Cell Imaging of Chromosome Dynamics During Differentiation Using the DeltaVision Microscope

1. Live-cell imaging conditions with any given microscope need to be adjusted, depending on the nature of the experiment and on the ESC line being used. Conditions need to be optimized to avoid phototoxicity issues and to ensure that the signal-to-noise ratios of the fluorescence signals are adequate. This can vary a lot depending on the ESC line and fluorescent protein being used. For example, in the case of imaging of TetO/TetR loci, taking only snapshots of cells to determine the position of

the TetO array can be done with rather high exposure times. Long-term live-cell imaging requires different imaging conditions (see below). The ESCs to be imaged can be located and focused on using the eyepiece and the bright-field mode of the microscope. Once cells are in focus, fluorescent light can be used to determine optimal imaging conditions (*see Note 12*). Avoid unnecessary exposure of cells with fluorescent light, which can induce phototoxicity and bleaching of the signal.

2. Determining optimal imaging conditions is crucial for the quality of the imaging experiment and the viability of the cells. The exposure time for live-cell imaging needs to be adjusted to minimize bleaching and phototoxicity. Depending on the excitation light and light source as well as the FPs used and their expression levels, phototoxic effects are positively correlated with the total exposure to light. The number of Z stacks will depend on the size of the object being imaged but should be minimized to reduce the total amount of light that the cells are exposed to during the acquisition. For our purposes (a single TetO array locus on the X chromosome), a step size of 0.3 μm was sufficient to faithfully detect the TetO array and measure 3D distances between the two loci on different X chromosomes. Depending on the size of the array and the signal-to-noise ratio in the cell line used, the step size might need to be adjusted. However, decreasing the step size means more images per Z stack, which will result in a higher amount of light exposure.
3. Use very low exposure times and test for phototoxicity (*see Subheading 3.3.5*) when performing long-term time-lapse live-cell imaging. Mouse ESCs are very sensitive to light and their differentiated derivatives even more so (*see Note 10*). The intervals between the acquisitions should be adapted to the total time of imaging. Typical examples of live-cell imaging with the DeltaVision are described below.
4. Figure 2a depicts a typical time-lapse acquisition performed over 3 h with 10-min intervals between acquisitions at the DeltaVision microscope. Acquired were Z stacks with a total number of 34 planes spaced by 300 nm (total stack size 10.2 μm). Acquisition time was 60 ms and the transmission filter was set to 32 %.
5. Figure 2b depicts a typical time-lapse acquisition performed over 30 min with 1-min intervals.

3.3.4 Long-Term Live-Cell Imaging Using the OMX Microscope

In order to increase speed of image acquisition, decrease the exposure time, and decrease phototoxicity during live-cell imaging of ESCs, OMX microscopy presents several advantages over the previous DeltaVision system we used. The DeltaVision is equipped with a standard metalloid lamp producing (depending on the filters used) a polychromatic excitation light. The OMX uses laser

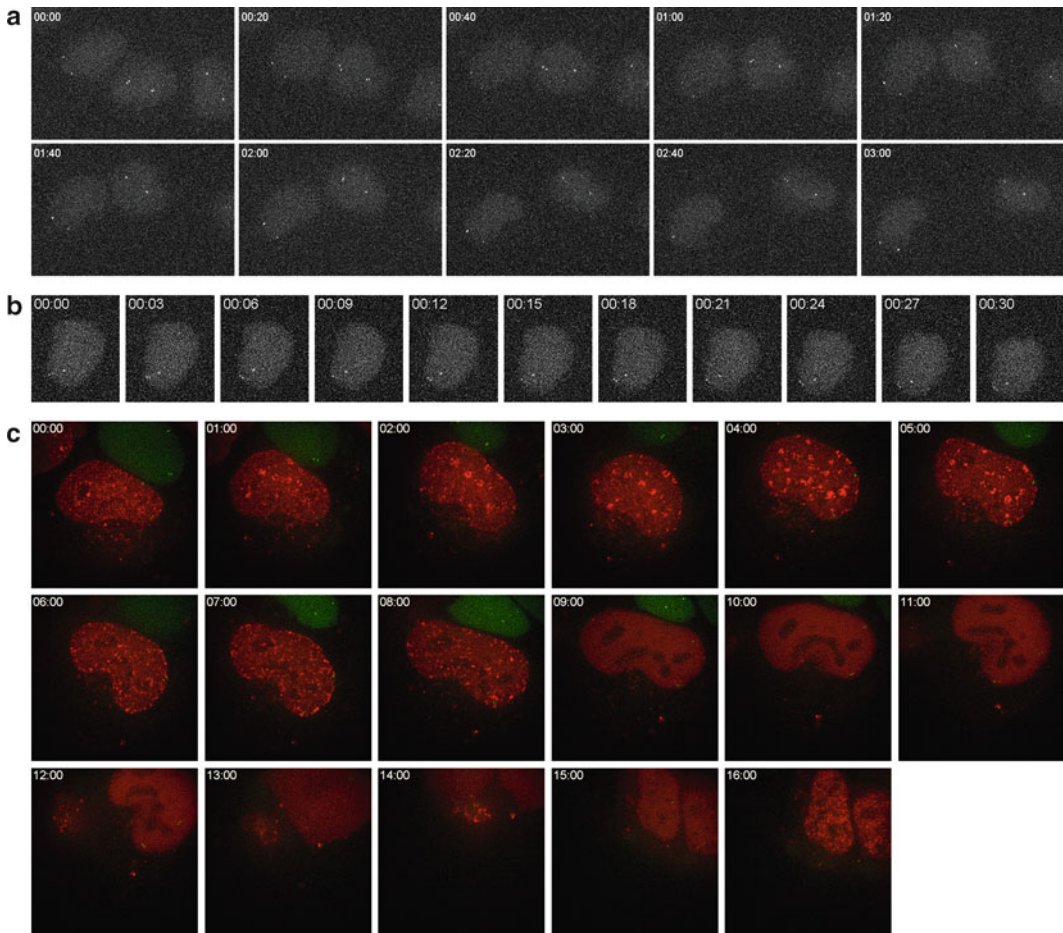


Fig. 2 (a) PGK 2TetO TetR-EGFP (undifferentiated) ESCs imaged for 3 h using the DeltaVision microscope with 10-min intervals (19 time points in total, shown is only every second time point). The displayed images are Z projections of stacks containing 34 planes with 300-nm spacing (total stack size 10.2 μm). The exposure time was set to 60 ms and the transmission filter set to 32 %. Nuclear shape can be determined from the TetR-EGFP background signal. The TetR-EGFP bound to one TetO array is represented by one spot. Depending on the cell cycle stage, two to four individual spots can be detected representing either unreplicated arrays, replicated arrays, or replicated arrays with two separated sister chromatids. The loci and the nuclei are highly dynamic. (b) PGK 2TetO TetR-EGFP (undifferentiated) imaged for 30 min using the DeltaVision microscope with 1-min intervals (31 time points in total, shown is only every third time point). The displayed images are Z projections of stacks containing 34 planes with 300 nm spacing (total stack size 10.2 μm). The exposure time was set to 60 ms and the transmission filter set to 32 %. Even during short-term acquisitions, one can observe highly dynamic sister chromatids and loci. Position and shape of ES cell nuclei also change during short-term acquisitions. (c) PGK 2TetO TetR-EGFP (undifferentiated) transfected with pBROAD3-mCherry-PCNA imaged for 16 h using the OMX microscope with 10-min intervals (96 time points, shown is only every sixth time point). The displayed images are Z projections of stacks containing 47 planes with 300-nm spacing (total stack size 14.1 μm). Acquisitions were made in the conventional mode using simultaneous acquisition. Exposure time was set to 20 ms for the green channel (488-nm laser, 0.1 % laser power) and 10 ms for the red channel (568-nm laser, 0.1 % laser power). The cell in the center of the images was in mid-S phase during the start of the acquisition, enters late S phase (time points: 2–8 h) before entering G_2 (time point 9 h). Cytokinesis occurs at time points 13–14 h. S phase is reinitiated in the daughter cells between time points 15 and 16 h

illumination, resulting in monochromatic excitation. In addition, the OMX contains multiple cameras (see specifications in Subheading 2) allowing for parallel multiwavelength acquisition in the different color channels. The OMX system is extremely stable, being encased in a vibration and dust-free environment, thus rendering far better conditions for long-term imaging. Parallel acquisition with multiple cameras and laser excitation allow reduction in exposure times and faster speed of acquisition using this system.

For long-term imaging, it is also extremely important to guarantee optimal and stable temperature and CO₂ conditions. To this end, the glass-bottom petri dish is covered with a heating lid in the observation room of the OMX. The sample holder, the stage, and the objective are also heated to avoid temperature gradient or variation. Temperature should be monitored using a temperature probe (see Subheading 2). Temperature fluctuations larger than 0.1 K from the optimal temperature should be avoided. Optimal CO₂ perfusion needs to be ensured using a gas perfusion system (see Subheading 2).

Figure 2c depicts a typical time-lapse two-color acquisition using the OMX, performed for 16 h with 10-min intervals. Z stacks consist of 48 planes, spaced by 300 nm (total stack size 14 µm). Exposure time was 20 ms for the green channel (488 nm) and 10 ms for the red channel (561 nm). Transmission was set to 0.1 % and the EMCCD camera gain to 300.

3.3.5 Assessing the Cell State After Long-Term Live-Cell Imaging

The color of the culture medium and cell morphology should always be recorded at the beginning and end of any live-cell imaging experiment. Useful indicators of possible phototoxicity include cell morphology, cell cycle, and cell division perturbations. In the ESC line we use as a model system, the nucleoli have a different background staining than the rest of the nucleus. Significant changes in nucleolar morphology can also be used as an indicator of phototoxic effects. Rounding up of the cells can indicate initiation of cell death. Creation of micronuclei is another indicator of phototoxicity. Prolongation of the cell cycle can also be indicative of phototoxic effects (induction of checkpoint). To assess for the degree of cell viability after imaging, the cells can either be fixed or assessed for DNA damage or cell cycle markers by immunofluorescence, or else they should be left on the microscope following imaging for several hours and checked for continued viability.

3.4 RNA FISH Following Live-Cell Imaging

Depending on the purpose of the experiment, cells can be fixed either directly after live-cell imaging or else following a given period of time. The protocols outlined below are described in more detail in ref. 11. An example of live-cell imaging of ESCs and their differentiated counterparts followed by fixation and RNA FISH is given in Fig. 3. Position of the cells was memorized using a petri dish with gridded coverslip (Fig. 3a; see also Subheading 3.3.2).

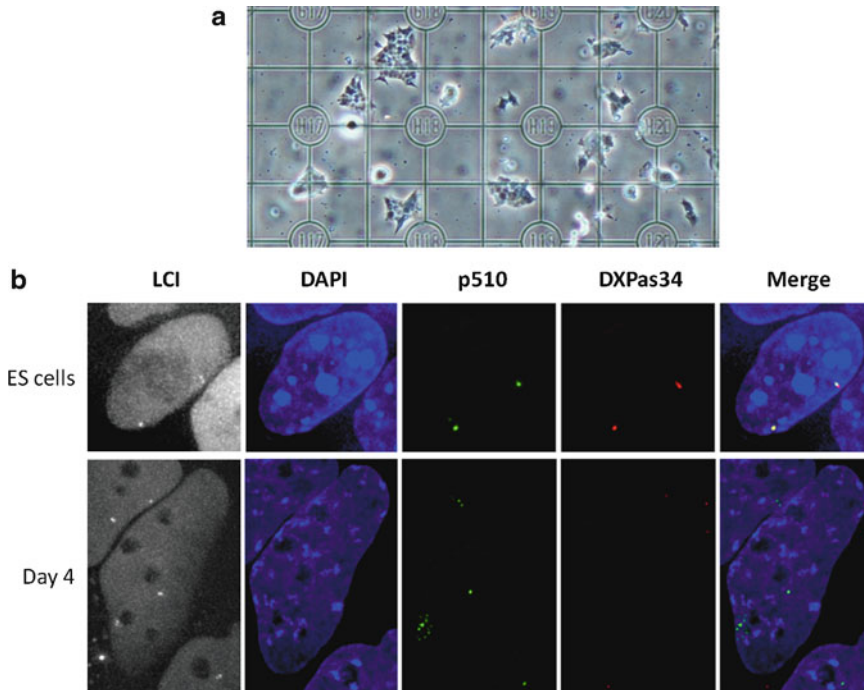


Fig. 3 (a) Small colonies (5–10 cells each) of the feeder-independent PGK12.1 ESC line in a 35-mm petri dish with 12-mm coverslip with grid. Positions and orientation of the colonies and cells in live-cell imaging can be memorized using the grid with the indicated letters and numbers. (b) Live-cell imaging (LCI) of PGK 2TetO TetR-mCherry cells with subsequent fixation and RNA FISH. ESCs or their differentiated counterparts (day 4 after LIF withdrawal) were imaged in a gridded petri dish directly before fixation. The same cells were imaged again after performing RNA FISH using a probe hybridizing to *Xist/Tsix* (p510) and *Tsix* only (DXPas34). In undifferentiated ES cells, *Tsix* is expressed biallelically from both X chromosomes (*upper panel*). At day 4 after LIF withdrawal, *Xist* expression has been monoallelically upregulated from one of the X chromosomes (*lower panel*)

3.4.1 Fixation and Permeabilization

1. Wash cells (on coverslips or in petri dishes) in pre-warmed 1× PBS.
2. Fix cells for 10 min in fixation solution at room temperature.
3. Wash cells three times with 1× PBS.
4. Permeabilize cells for 4–5 min in ice-cold permeabilization solution on ice.
5. Wash cells three times with 70 % ethanol (only for RNA and DNA FISH).
6. Store coverslips in 6-well plates or petri dishes in 70 % ethanol at -20°C (*see Note 13*).

3.4.2 FISH DNA Probe Labeling

1. For a 50- μl reaction mix, 1–2 μg of plasmid or BAC/plasmid DNA is mixed with water to 17.5 μl , 2.5 μl of 0.2 mM SR-, SG-, Cy5-dUTP, 10 μl 10 mM each dNTP mix (dGTP, dATP,

dCTP), 5 μ l 10 mM dTTP, 5 μ l 10 $\times$ nick translation buffer, and 8–10 μ l nick translation enzyme.

2. Incubate for 16 h at 15 °C in the dark.
3. Inactivate the reaction by freezing at –20 °C. Probes can be stored for up to a few weeks at –20 °C.

3.4.3 Probe Preparation

1. 0.1 μ g probe per coverslip and Cot-1 DNA (competition is required for most probes as they can contain repeat sequences that will cross hybridize and increase background unless competed away prior to hybridization) are precipitated by addition of 10 μ g salmon sperm DNA, 1/10 volume 3 M sodium acetate pH 5.2, and three volumes ethanol at 16,100 $\times g$ and 4 °C for 30 min.
2. Wash precipitate with 75 % ethanol and spin down at 16,100 $\times g$ and 4 °C for 5 min.
3. Dry for 2 min in a concentrator/speed vac.
4. Resuspend in an appropriate volume of formamide (half the volume required for hybridization).
5. Incubate for at least 30 min at 37 °C and 1,400 rpm in a Thermomixer.
6. Denature for 10 min at 75 °C in a Thermomixer.
7. Quench on ice, or if competition is to be performed, put directly at 37 °C for at least 30 min in a Thermomixer.
8. Mix the probe solution with an equal volume of 2 $\times$ hybridization solution.

3.4.4 Hybridization and Washes

1. Dehydrate coverslips (in wells or petri dishes) by sequential washing in 1 $\times$ 80 %, 1 $\times$ 95 %, and 2 $\times$ 100 % ethanol for 5 min.
2. Dry coverslips or petri dishes completely.
3. Hybridization using coverslips: Spot the 12- μ l probe hybridization mix (*see* **step 8**, Subheading 3.4.3), without making bubbles, onto a slide and lower the coverslip, with cells facing into the hybridization mix. Continue with **step 5**.
4. Hybridization in live-cell imaging petri dishes: Spot an appropriate amount of hybridization solution directly onto the coverslip in the bottom of the petri dish. Place a 12-mm glass coverslip on top of the hybridization solution to prevent evaporation. Continue with **step 5**.
5. Place slides or petri dishes in a humid chamber (tissue paper soaked in 50 % FA/2 $\times$ SSC) and incubate at 37 °C overnight.
6. Post-hybridization washes (slides): Add 1 ml of 50 % FA/2 $\times$ SSC onto the coverslip on the slide to loosen it, remove the coverslip carefully from the slide (taking care not to scrape the cells), and place it with cells facing upwards, into a 6-well plate containing 50 % FA/2 $\times$ SSC. Continue with **step 8**.

7. Post-hybridization washes (petri dishes): Add 1 ml of 50 % FA/2× SSC into the petri dish to loosen the coverslip and remove the 12-mm coverslip from the bottom of the petri dish using a fine pair of forceps taking care not to scrape the cells. Continue with **step 8**.
8. Perform three washes, each for 7 min at 42 °C, with pre-warmed 50 % FA/2× SSC pH 7.2–7.4
9. Perform three washes with pre-warmed 2× SSC for 5 min each at 42 °C.
10. Counterstain nuclei by washing in 2× SSC with DAPI for a minimum of 3 min at room temperature.
11. Wash three times with 2× SSC.
12. Mounting of coverslips: Spot an appropriate amount of mounting medium on a slide and place the coverslip on top of the drop with cells facing down (avoid bubbles). Wipe off excess mounting solution and seal the coverslip with a small amount of nail polish.
13. Mounting of petri dishes: Spot an appropriate amount of mounting medium on the coverslip in the bottom of the petri dish. Place an 18-mm glass coverslip on top of the mounting medium (avoid bubbles). Wipe off extensive mounting solution and seal coverslip with a small amount of nail polish.

3.5 Protocol for DNA FISH

The fixation of samples prior to DNA FISH is the same as for RNA FISH. Coverslips or petri dishes with coverslip stored in 70 % ethanol can be used for DNA FISH.

3.5.1 RNase Treatment

1. After dehydration (through 80, 95, and 100 % ethanol series as described above) of coverslips or petri dishes with glass bottom (*see* Subheading 3.4.4), add 1–2 ml of 2× SSC supplemented with 0.1 mg/ml RNase A and 10 U/ml RNase H to the coverslip and incubate for 1 h at 37 °C.
2. Wash 3× with 2× SSC.

3.5.2 Denaturation

1. Preheat 50 % FA/2× SSC (pH 7.2–7.4) to 80 °C in a water bath.
2. Preheat a hybridization oven to 80 °C.
3. Add the preheated 50 % FA/2× SSC to the coverslips and incubate in a hybridization oven at 80 °C for 37–40 min (*see* **Note 14**).
4. Quickly remove the denaturation solution from the coverslip and add ice-cold 2× SSC.
5. Wash 2× with ice-cold 2× SSC and keep on ice until hybridization.

3.5.3 Hybridization and Washes

Hybridization and washes are performed as described above (*see* Subheading 3.4.4). Note that for DNA FISH, hybridization and washes are performed at higher temperatures (e.g., 42 °C for the hybridization and 45 °C for the washes).

3.6 Immunofluorescence (RNA or DNA FISH) Protocol

3.6.1 Fixation and Blocking

1. Fixation is performed as described in Subheading 3.4.1 except for the last washing step in 70 % ethanol, instead of washing with 70 % ethanol wash 3× in 1× PBS.
2. Blocking is performed in 1 % BSA in PBS for 15 min at room temperature.

3.6.2 Antibody Incubation

For cells on coverslips:

1. Dilute primary antibody in antibody dilution buffer and apply 50 µl of the antibody solution onto a clean glass slide.
2. Place coverslip cells down onto each of the drops and hybridize for 40 min at room temperature.
3. Wash 3× in 1× PBS for 5 min at room temperature on a rotating shaker.
4. Dilute the secondary antibody in antibody dilution solution and apply 50 µl onto a clean glass slide.
5. Put coverslips cells down onto each of the drops and hybridize for 40 min at room temperature in the dark.
6. Wash 3× in 1× PBS for 5 min at room temperature on a rotating shaker in the dark.
7. Fix for 10 min at room temperature with fixation solution.
8. Wash 3× quickly in 1× PBS.
9. Mount coverslips (IF only) or wash 2× in 2× SSC and proceed with **step 4** of Subheading 3.4.4 for IF-RNA FISH or Subheading 3.5.2 for IF-DNA FISH.

For cells on petri dishes:

1. All solutions are applied directly to the coverslip at the bottom of the petri dish. Incubations and washes are performed as described above.

4 Notes

1. The exact concentration of drug to be used for any particular ESC line should always be verified prior to the experiment, by testing various concentrations and evaluating kinetics and extent of cell death. Different ESC lines can show substantial variations in drug sensitivity.

2. Clones should be selected based on high signal-to-noise ratios, where the two TetO loci are visible in the majority (>90 %) of cells and for which minimal mosaicism is seen.
3. Glass coverslips: Make sure the brand and batch of glass coverslips used (see materials for examples) are suited for culturing ES cells as well as for the live-cell imaging device, the objective and microscope you use to achieve optimal imaging results.
4. Pre-coating of coverslips and dishes: We have noted that certain glass surfaces and dishes are suboptimal for feeder-independent ESC cultures for live-cell imaging. Cells need to grow in relatively thin colonies, with one or at most two cell layers. 0.1 % gelatin is the usual coating agent used—however, if colonies are too round and multilayered for optimal imaging, pre-coating with poly-d-lysine or fibronectin can be used. It should be verified that poly-d-lysine or fibronectin coating does not change the properties of the ES cells (other than cell shape) by checking for pluripotency marker staining; the differentiation kinetics of ES cells should also be compared to those cultured on standard gelatin coating.
5. Cell density: The optimal density of cells seeded at day 0 or at day -1 should be determined in advance, as it is different for every ESC line and differentiation condition used. Titration experiments should be performed on the coverslips or in the petri dishes and the pre-coating used for the actual experiment to determine the initial cell density needed to gain sub-confluent and optimal density at the day of imaging.
6. Medium for live-cell imaging: Tolerance to different media needs to be tested for individual ESC lines. Better imaging results can often be obtained using medium without phenol red. Phenol red-containing medium can induce changes in ESC morphology, TetO array signal detection, and cell viability in our experience.
7. Ludin live-cell imaging chamber: Make sure that the coverslip has no scratches or breaks and that the isolation rubber is placed on the coverslip edges and that both are even before carefully screwing the top part on the Ludin chamber. Carefully clean the bottom of the coverslip after assembly of the chamber with a tissue to wipe off remaining medium and cells.
8. Antibiotics: For long-term imaging, we recommend the use of antibiotics in the medium, especially when using a Ludin chamber or an open petri dish.
9. CO₂ perfusion system: Make sure that the gas is pre-warmed and pre-humidified. Dry gas will lead to fast evaporation of medium.

10. 35-mm petri dishes with glass coverslip: The use of petri dishes with a lid limits evaporation as well as the risk of bacterial or fungal contamination of the medium. If a lid on the dish cannot be used, due to a special heating or CO₂ perfusion system, evaporation of the medium can be prevented by covering it with biology grade, sterile-filtered paraffin (or mineral oil). In our experience this does not interfere with ESC growth or morphology.
11. Orientation of the grid: Prior to the live-cell imaging, note down the orientation of the grid (using the numbered fields) on the microscope to find cells more easily after fixation and have them in the same orientation for imaging.
12. UV filter/neutral density filters: Use such filters as much as possible, to decrease phototoxicity.
13. Storage of petri dishes and coverslips: After fixation coverslips can be stored for several months in 70 % ethanol at -20 °C. Make sure that the petri dish or 6-well plate containing the coverslips is thoroughly sealed with parafilm to prevent evaporation of the ethanol.
14. Denaturation for DNA FISH: Denaturation time for optimal DNA FISH results differs greatly between cell types and even for the same ES cell line at different stages of differentiation. The genomic locus being detected can also vary considerably. Test run DNA FISH should be performed to ensure that the conditions are optimal for a given cell type and locus.

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