Chapter 12

Measuring the Dynamics of Chromatin Proteins During Differentiation

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Abstract

Chromatin-protein interactions are important in determining chromosome structure and function, thereby regulating gene expression patterns. Most chromatin associated proteins bind chromatin in a transient manner, with residence times on the order of a few seconds to minutes. This is especially pertinent in mouse embryonic stem cells (ESCs), where hyperdynamic binding of chromatin associated proteins to chromatin is thought to regulate genome plasticity. In order to quantitatively measure binding dynamics of such chromatin proteins in living cells, a combination of GFP-fusion proteins and photobleaching-based assays such as fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are advantageous over other existing biochemical assays, because they are applied in living cells at a single cell level. In this chapter we describe a detailed protocol for performing FRAP and FLIP assays for measuring structural chromatin protein dynamics such as Heterochromatin Protein 1 (HP1) and linker histone H1 in mouse ESCs and during ESC differentiation.

Key words GFP-fusion proteins, FRAP, FLIP, Chromatin binding proteins, Embryonic stem cells, Live imaging

1 Introduction

Gene expression, the fundamental cellular process, is chiefly regulated by chromatin structure and its interaction with chromatin binding proteins [1]. Apart from the core histones themselves, which are strongly associated with DNA, the interactions of proteins with chromatin are highly dynamic in nature [2]. This dynamicity is particularly high in embryonic stem cells (ESCs), where hyper-dynamic binding of structural chromatin proteins is considered to be partly responsible for genome and functional plasticity [3]. Therefore, studying the dynamic association between chromatin proteins and chromatin is very informative, especially since the methods that are used to decipher the binding kinetics are applied in single, living cells. This is even more pertinent when dealing with ESCs, since, as noted above, chromatin proteins,

Yaron Shav-Tal (ed.), *Imaging Gene Expression: Methods and Protocols*, Methods in Molecular Biology, vol. 1042,
DOI 10.1007/978-1-62703-526-2_12,

including heterochromatin protein 1 (HP1), linker histone H1 and core histones, all have a higher turnover rate (dynamic exchange rates) on chromatin, compared to normal differentiated cells [1, 3]. To study the dynamic association of chromatin binding proteins with chromatin, photobleaching-based assays such as Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss In Photobleaching (FLIP) are normally used. These assays provide powerful spatial and temporal information regarding the protein's turnover rate (on/off rates), its mobile fraction, immobile fraction and whether or not it shuttles between compartments within cells or nuclei.

To carry out photobleaching-related experiments, the desired gene is fused to fluorescent proteins such as GFP, YFP, mCherry etc., and expressed in living cells [4]. Additionally, GFP gene tagging in BACs using recombineering [5] or CD-tagging of endogenous genes directly with GFP/YFP exons can also be used for this purpose [6, 7]. These methods are preferable, as the fusion protein is driven by an endogenous promoter, but usually are unavailable. It should be noted that a fluorescent tag could alter the normal function of the protein. Thus, to ensure precision, the protein should be monitored and compared to a normal protein.

In FRAP assays, after successful expression of the fusion protein of interest inside the cells, an intense focused laser beam is used to bleach a relatively small region of interest (ROI), where the laser wavelength is selected according to the fluorescent protein used for fusion. Time lapse imaging is then used to monitor the recovery of the bleached molecules, which are replaced with unbleached molecules following the irreversible photobleaching event. FLIP is a complementary technique that measures signal decay rather than recovery. It not only determines the protein's mobility, but also by bleaching one compartment and measuring the fluorescence decay in another determines whether the protein shuttles between different compartments [8–12].

For a successful FRAP experiment, certain conditions must be fulfilled. First, the fluorescent signal to be bleached must be clearly visible over any background. Second, the photo-bleaching should be fast enough so that recovery during the bleaching itself is negligible, allowing good temporal resolution and enabling to calculate the recovery half-time. To achieve this, the laser used for photobleaching must be powerful enough (usually in the range of 50 mW). A laser with sufficient power ensures high spatial resolution and allows the bleaching of small, submicron, cellular areas. Finally, it is essential to maintain the homeostasis of the cells. This can be achieved by mounting an environmental chamber on the confocal microscope. To minimize photodamage due to prolonged imaging, a spinning disk confocal microscope equipped with a photobleaching module is recommended.

In the present chapter we describe the basic requirements and methodology to perform FRAP and FLIP assays, particularly in mouse ESCs, for the study of chromatin protein dynamics in undifferentiated cells and during ESC differentiation.

2 Materials

2.1 Cloning Your Gene of Interest (GOI) into a Vector Expressing a Fluorescent Protein

1. Plasmid: For N-terminal fusions (the GFP will be at the C-terminal end), use pEGFP-N1 plasmid (*see* **Note 1**) and for C-terminal fusions (the GFP will be at the N-terminal end) use pEGFP-C1 plasmid (*see* **Note 2**) (all available from Clontech).
2. Restriction enzymes for which a recognition sequence is present at the multiple cloning site (i.e. BglII, XhoI, HindIII, BamHI).
3. 5 units/μl T4 DNA ligase.
4. TOP 10 chemically competent *E. coli* or equivalent for transformation.

2.2 Cell Culture and Plasmid Transfection for Transient Expression of the GFP Fusion Protein

1. Dulbecco's Modified Eagle's Medium (DMEM).
2. ESC-grade fetal bovine serum (FBS).
3. 1 mM sodium pyruvate.
4. 0.1 mM nonessential amino acids.
5. 0.1 mM β-mercaptoethanol.
6. 1,000 U/ml leukemia inhibitory factor (LIF).
7. 0.25 % trypsin for detaching cells from tissue culture plate.
8. Phosphate-buffered solution (PBS) for washing cells.
9. GFP fusion plasmid DNA.
10. Opti-MEM reduced serum free medium.
11. TransIT-LT1 transfection reagent (Mirus).
12. R1 mouse embryonic stem cells (or equivalent) and mouse embryonic fibroblasts.
13. 0.5 μM All-trans Retinoic Acid (ATRA).

2.3 Time-Lapse Live-Cell Imaging of Fluorescent Protein Dynamics in Living Cells

1. Confocal microscope of choice (laser scanning or spinning disk confocal) equipped with a 60× NA = 1.4 (or higher) oil objective. We recommend a Revolution spinning disk confocal system (Andor) with the Yokogawa CSU-X spinning disk head. This system is equipped with a dual capacity to photobleach with a point scanning system and to switch back the laser light to collect images using the spinning disk. GFP, YFP and mCherry are the fluorescent proteins generally used in FRAP/FLIP experiments. A ~488 nm laser is required for GFP and

YFP, and a ~560 nm laser is required for mCherry. Solid state lasers are preferable but gas-medium lasers are also commonly used. Since experiments are conducted on living cells, an environmental microscope chamber is required to maintain humidity, CO_2 and temperature (*see* **Note 3**). Photobleaching is performed under maximal laser intensity (100 %) and imaging under minimal intensity that allows proper signal to noise ratio (around 10 %) to prevent phototoxicity and photobleaching during imaging.

2. 8-well μ-Slides (ibidi; Munich, Germany) or chambered cover glasses (Lab-Tek; Rochester, NY) or glass-bottom culture dishes (MatTek; Ashland, MA).
3. Image analysis software such as: Imaris (http://www.bitplane.com/), Metamorph (Molecular Devices, Downingtown, PA), or ImageJ (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/).

3 Methods

3.1 Construction of the Expression Vector Containing Your Gene of Interest (GOI) Fused with a Fluorescent Protein

Clone your gene of interest (GOI) into the expression vector selected (pEGFP-C1 or pEGFP-N1, see above for details). To express a fluorescently tagged protein, the coding region of the gene should be inserted in frame with a vector containing a fluorescent protein of choice such as GFP, YFP or mCherry etc. (*see* **Notes 2** and **3**). The presence of an antibiotic resistance gene in the vector will help in generating stable cell lines.

3.2 Transfecting ES Cells

1. Coat the 8-well μ-Slides with gelatin and let stand for 15–30 min.
2. Aspirate gelatin and seed 22,000 MEFs/well in 250 μl total volume of DMEM (supplemented with 10 % FBS). Grow the cells in a tissue culture incubator (37 °C, 5 % CO_2). MEFs are required to maintain ES cell pluripotency at the undifferentiated state.
3. After 6 h, aspirate the media, and seed 15,000 R1 ES cells/well in each MEF-coated well in 250 μl ESC media (supplemented with 10 % ESC-grade FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 1,000 U/ml LIF), to reach 50 % confluence the following day.
4. After 18 h, replace the ESC media with 250 μl/well of fresh ESC media.
5. In a 1.5-ml Eppendorf tube, dilute 10 μl TransIT-LT1 transfection reagent (Mirus) in 100 μl reduced serum free media (Opti-MEM). Mix gently with pipette and incubate at room temperature for 5–20 min.

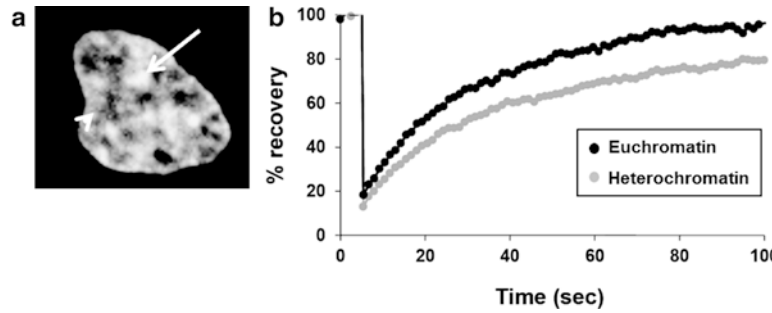

Fig. 1 (**a**) A representative embryonic stem cell expressing H1e-GFP fusion protein, where heterochromatin (*arrow*) and euchromatin (*arrowhead*) are readily distinguishable. (**b**) A representative FRAP curve of H1e-GFP fusion protein in embryonic stem cells, where heterochromatin (*black*) and euchromatin (*grey*) were analyzed separately. Shown is an average of 20 cells

6. Add 1.5 μg GFP fusion plasmid DNA to the diluted transIT-LT1 reagent and mix by gentle pipetting and incubate at room temperature for 15–30 min.
7. Add 13.5 μl of transfection complexes to each well containing cells and media. Swirl the 8-well μ-Slides to ensure even dispersal.
8. After 24 h, replace the old ESC media with 250 μl of fresh ESC media.
9. For differentiation, treat ESCs with 1.0 μM Retinoic Acid for 2–5 days in ESC media without LIF, and repeat experiments in differentiating cells (*see* **Note 4**).

3.3 Performing FRAP and FLIP

1. Mount the 8 well μ-slide inside the microscope. Find the cells expressing the fluorescently labeled protein and adjust the imaging conditions such that clear fluorescence signal can be detected with minimal background (Fig. 1a). Ensure proper localization of the fluorescent proteins. When expression levels are too high, "spilling" of the protein into additional cellular compartments, such as the nucleolus, can occur. Long-term exposure to fluorescent lamps and to high intensity lasers should be avoided to reduce photobleaching of the fluorescent proteins and photodamage to the cells in general.
2. Experimental parameters required for time-lapse imaging including exposure time, number of fields, and the number of repeats should be adjusted as needed. For highly mobile proteins, i.e. HP1, use 3–4 frames/s for 20–40 s. For proteins with intermediate mobility, i.e. H1, use 1–2 frames/s for around a minute. For immobile proteins, i.e. core histones, use 6–12 frames/min for 5–10 min. ESCs are notoriously difficult

to image for extended periods due to motion. Light intensity and exposure time used in the experimental procedure should be as low as possible to prevent photobleaching, but sufficient to distinguish signal over background. Do not use frame averaging. The quality of the image itself is secondary.

3. For FRAP, set the following protocol: collect 3–5 frames before photobleaching, then photobleach, then collect an additional 60–120 images at the desired intervals according to the protein investigated. Bleach spot should be minimal. Appropriate photobleaching reveals a black hole in the GFP fluorescence signal that slowly "recovers" or fills up with unbleached fluorescent proteins from the surroundings. Repeat for 20–30 cells in three different experiments.
4. For FLIP, set the following protocol: Collect 3–5 frames before bleaching, then repeatedly bleach the same spot while simultaneously collecting images. In FLIP experiments, the decay in fluorescence is slower than the recovery in fluorescence in FRAP experiments and the interval between images should be longer. For proteins with intermediate mobility, bleach and collect image every 5 s, and for highly mobile proteins bleach and collect image every 1–2 s. Repeat for 20–30 cells in three different experiments (*see* **Note 5**).

3.4 FRAP and FLIP Data Analysis

1. FRAP. In each one of the FRAP frames collected (before and after bleaching), measure the fluorescence intensity in the bleached region (ROI_b), in a non-bleached region within the same cell (ROI_{nb}) and in a background region outside the cell (ROI_{bg}). For each frame, use the following formula:

$$F = \left(ROI_b - ROI_{bg}\right) / \left(ROI_{nb} - ROI_{bg}\right) / \left({}_{pb}ROI_b - pbROI_{bg}\right) / \left({}_{pb}ROI_{nb} - {}_{pb}ROI_{bg}\right),$$

where pb denotes "pre-bleached". The approximate value must be 1 for the first few pre-bleached images. The first image after the bleach gives the value of the bleach depth which is subtracted from 1. The experiment is repeated for every cell for 20–30 cells (*see* **Note 6**). A representative FRAP curve (an average of ten cells) is shown in Fig. 1b.

2. FLIP. Here, the fluorescence intensity is a measure of the non-bleached nuclear area (ROI_{nb}) and the background area (ROI_{bg}). The calculation of the FLIP data is similar to the FRAP curve, where only the analyzed area (ROI_{nb}) must be different compared to the actual bleached area and is not used in the calculations $(ROI_{nb} - ROI_{bg})/({}_{pb}ROI_{nb} - {}_{pb}ROI_{bg})$. Here, a neighboring cell (ROI_n) can be used for normalization according to the formula:

$$F = \left(ROI_{nb} - ROI_{bg}\right) / \left(ROI_n - ROI_{bg}\right) / \left({}_{pb}ROI_{nb} - {}_{pb}ROI_{bg}\right) / \left({}_{pb}ROI_n - {}_{pb}ROI_{bg}\right).$$

Using curve fitting, one can estimate with good proximity the mobile fraction, the immobile fraction and the half-maximum. The distance between the bleach depth and the recovered signal on the Y-axis refers to the mobile fraction, especially when the kinetics reaches a plateau. The immobile fraction is the distance between the recovered signal and the pre-bleach (100 %) signal on the Y-axis. The immobile fraction represents the molecules that are tightly bound and are not exchanged within the timescale of the experiment.

Mathematical models are available to fit the data. If only a single population of molecules is present, all sharing the same mobility parameters, the data can fit a single exponential equation, where t is time, A is the mobile fraction, $1 - A$ is the immobile fraction and k_{off} is the dissociation constant. In most cases, we find that the data fits a two component exponential equation $I(t) = A_1(1 - e^{-K_{1\text{off}} \cdot t}) + A_2(1 - e^{-K_{2\text{off}} \cdot t})$, indicating two mobile populations with different kinetics.

4 Notes

1. Use pEGFP-N1, pEGFP-N2 or pEGFP-N3 according to the available reading frame.
2. Use pEGFP-C1, pEGFP-C2 or pEGFP-C3 according to the available reading frame.
3. If only short-term experiments are planned, an air blower which maintains a constant temperature is also optional. In this case, it is preferable to use a pH-maintaining buffer.
4. Many different differentiation protocols are available. The advantage of using retinoic acid, although the resulting cells are not completely homogenous, is that the cells are grown as monolayers and do not require transfer.
5. Since the shape and size of the bleached region influences the recovery dynamics, identical protocols must be followed for each protein and experiments comparing between different conditions should be carried out on the same day since laser power and other conditions may vary.
6. An alternative to FRAP is inverse-FRAP (or iFRAP), where the entire nucleus is bleached except for the ROI and the fluorescence decay is measured at the ROI. This can be used to measure k_{on}.

Acknowledgements

We thank the Abisch Frenkel Foundation, the Israel Cancer Research Foundation (ICRF), the Human Frontiers Science Foundation (HFSP), the Israel Science Foundation (ISF 657/12;

1252/12), the Israel Ministry of Science, and the European Research Council (ERC-281781) for financial support. AH is Marie Curie Nucleosome4D fellow.

References

1. Meshorer E, Yellajoshula D, George E et al (2006) Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. Dev Cell 10:105–116
2. Phair RD, Misteli T (2000) High mobility of proteins in the mammalian cell nucleus. Nature 404:604–609
3. Melcer S, Hezroni H, Rand E et al (2012) Histone modifications and lamin A regulate chromatin protein dynamics in early embryonic stem cell differentiation. Nat Commun 3:910
4. Nissim-Rafinia M, Meshorer E (2011) Photobleaching assays (FRAP & FLIP) to measure chromatin protein dynamics in living embryonic stem cells. J Vis Exp 52:e2696
5. Poser I, Sarov M, Hutchins JR et al (2008) BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals. Nat Methods 5:409–415
6. Sigal A, Danon T, Cohen A et al (2007) Generation of a fluorescently labeled endogenous protein library in living human cells. Nat Protoc 2:1515–1527
7. Cohen AA, Geva-Zatorsky N, Eden E et al (2008) Dynamic proteomics of individual cancer cells in response to a drug. Science 322: 1511–1516
8. Ellenberg J, Siggia ED, Moreira JE et al (1997) Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. J Cell Biol 138:1193–1206
9. Dundr M, Misteli T (2003) Measuring dynamics of nuclear proteins by photobleaching. Curr Protoc Cell Biol Chapter 13, Unit 13 5
10. Phair RD, Misteli T (2001) Kinetic modelling approaches to in vivo imaging. Nat Rev Mol Cell Biol 2:898–907
11. Mueller F, Mazza D, Stasevich TJ et al (2010) FRAP and kinetic modeling in the analysis of nuclear protein dynamics: what do we really know? Curr Opin Cell Biol 22:403–411
12. Lenser T, Weisshart K, Ulbricht T et al (2010) Fluorescence fluctuation microscopy to reveal 3D architecture and function in the cell nucleus. Methods Cell Biol 98:2–33