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Parsing stem cell lineage development using high content image analysis of epigenetic spatial markers

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Abstract

This unit describes a protocol for acquiring and analyzing high-content super resolution images of human stem cell nuclei for the characterization and classification of the cell differentiation paths based on distinct patterns of epigenetic mark organization. Here, we describe the cell culture, immunocytochemical labeling, super resolution imaging parameters, and MATLAB based quantitative image analysis approaches for monitoring human mesenchymal stem cells (hMSCs) and human induced pluripotent stem cells (hiPSCs) as the cells differentiate towards various lineages. Although this protocol uses specific cell types as examples, this approach could be easily extended to a variety of cell types and nuclear epigenetic and mechanosensitive biomarkers that are relevant to specific cell developmental scenarios.

Introduction

The organization and expression of specific biomarkers can serve as probes of long-term cell functions, as the organization, distribution and overall expression of specific mechanosensitive proteins can serve as surrogate reporters of the state of the cell. In particular, the spatiotemporal expression of epigenetic markers play a large role in shaping chromatin structural dynamics, which in turn regulate the gene transcription and subsequent behavior of different cells. Some epigenetic marks have been linked with cellular differentiation towards specific phenotypes, as well as cellular reprogramming to a pluripotent state.

Current methods for elucidating the epigenome are mostly population based, and involve cell lysis to isolate chromatin for immunoprecipitation or proteomic interrogation via mass spectrometry. These approaches are unable to identify spatial coordinates for dynamic chromatin marks. We therefore developed an optical image based technique that preserves chromatin architectural information at a single cell level and can thus provide insights into how the structural organization of chromatin correlates with gene expression.

Here, we describe a protocol to monitor and predict the differentiation of human mesenchymal stem cells towards specific lineages using two key epigenetic markers, the trimethylation of lysine residues 4 and 27 on histone 3 (H3K4me3 and H3K27me3). In the detailed protocols below, we describe the culture and directed differentiation of human mesenchymal stem cells towards adipogenic (fat) and osteogenic (bone) lineages (basic

protocol 1), immunocytochemical labeling of nuclear markers (basic protocol 2), high content image acquisition using super-resolution nanoscopy (basic protocol 3), and image processing and analysis using ImageJ (basic protocol 4). (Figure 1)

Basic protocol 1: *Cell culture and directed differentiation of human mesenchymal stem cells*

This protocol describes the *in vitro* culture, maintenance and directed differentiation of human mesenchymal stem cells (hMSCs) towards adipogenic and osteogenic lineages.

Note: The following protocol must be conducted in a class II biosafety cabinet, with proper sterile techniques used.

Note: All incubations are performed in a humidified incubator at 37 °C, 5% CO₂

Materials

Frozen vial of hMSCs, purified by FACS

PBS w/o Ca⁺⁺/Mg⁺⁺ (Lonza, Cat No. 17-516F)

0.25% Trypsin-EDTA (Thermo-Fisher, Cat No. 25200114)

Basal Mesenchymal Stem Cell Maintenance Media: Minimum Essential Medium (MEM)- α without deoxy- nor ribo-nucleosides (Gibco, CA), supplemented with 10% fetal bovine serum (FBS) (Gibco, CA) and 100 units/mL penicillin plus 100 μ g/mL streptomycin (Gibco, CA).

Osteogenesis Induction Media: MEM- α without deoxy- nor ribo-nucleosides, supplemented with 10% FBS, 10 mM β -glycerophosphate (Sigma-Aldrich, Saint Louis, MO), 0.1 μ M dexamethasone (Sigma-Aldrich, Saint Louis, MO), 0.5 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich, Saint Louis, MO) and 100 units/mL penicillin plus 100 μ g/mL streptomycin.

Adipogenesis Induction Media: MEM- α without deoxy- nor ribo-nucleosides, supplemented with 10% FBS, 1 μ M dexamethasone, 0.2 mM indomethacin (Sigma-Aldrich, Saint Louis, MO), 0.01 mg/ml insulin (Sigma-Aldrich, Saint Louis, MO), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, Saint Louis, MO) and 100 units/mL penicillin plus 100 μ g/mL streptomycin.

Adipogenic Maintenance Media: MEM- α without deoxy- nor ribo-nucleosides, supplemented with 10% FBS, 0.01 mg/mL insulin (Sigma-Aldrich, Saint Louis, MO) and 100 units/mL penicillin plus 100 μ g/mL streptomycin.

75 cm² culture area cell culture treated flasks (Thermo-Fisher Scientific)

Protocol Steps

Expansion and maintenance of hMSCs

1. Quick thaw frozen vial of hMSCs in a 37° C water bath

Bone marrow derived hMSCs can be obtained from various sources, but should be comprehensively characterized and sorted by FACS for the cell surface markers CD3, CD11b, CD14, CD19, CD29, CD34, CD36, CD44, CD45, CD49bcd, CD59, CD73a, CD79a, CD90, CD105, CD106, CD117, CD146, CD147, CD166, CD184, CD271, HGFR(c-Met), HLA-I, HLA-II, PODXL)

2. Transfer vial contents into a 15 mL conical tube containing 9 mL of RT basal mesenchymal stem cell maintenance media.
3. Centrifuge conical tube at 600 RCF for 5 minutes
4. Aspirate supernatant, and resuspend in 1 mL of basal mesenchymal stem cell maintenance media.
5. Transfer resuspended cells in a T75 flask containing 10 ml of basal mesenchymal stem cell maintenance media.
6. Allow cells to attach for a minimum of 6 hours.
7. Culture hMSCs by cycling fresh media every 48 hours until they reach 50% confluency. (Figure 2)
8. Remove medium and wash cells 1X with pre-warmed PBS w/o Ca⁺⁺/Mg⁺⁺ (Lonza)
9. Remove PBS and add Trypsin-EDTA (0.25%) (Sigma) to incubate for 5 minutes.
10. Gently tap expansion flask and verify cell detachment under a light microscope.
11. Add equal volume of basal mesenchymal stem cell maintenance media to neutralize trypsin.
12. Transfer contents of flask to a 15 mL conical tube and centrifuge at 600 RCF for 5 minutes.
13. Aspirate supernatant, and resuspend in 1 mL of basal mesenchymal stem cell maintenance media.
14. Passage cells at a seeding density of 5,000 cells/cm² into a T75 flask containing a total of 10 ml of basal mesenchymal stem cell maintenance media.

For hMSC maintenance, repeat steps 6–14.

Directed differentiation of hMSCs toward adipogenic hMSCs

1. Passage cells at a seeding density of 21,000 cells/cm².
2. Change media once every 48 hours until cells reach 100% confluency.
3. Once cells reach 100% confluency, begin differentiation by replacing media with adipogenic induction media, and incubating for 72 hours.
4. Replace adipogenic induction media with adipogenic maintenance media, and incubate for 48 hours.

5. Repeat steps 3–4 three times (i.e. three cycles of adipogenic induction media followed by adipogenic maintenance media).

Directed differentiation of hMSCs toward osteogenic hMSCs

1. Passage cells at a seeding density of 3,000 cells/cm².
2. Allow cells to attach for 24 hours, and replace media with osteogenesis induction media.
3. Replace media every 72 hours over a two-week period.

Basic protocol 2: *Immunocytochemical labeling of Epigenetic Markers (H3K4me3 and H3K27me3)*

This protocol describes the preparation of cells for high resolution imaging of nuclear biomarkers. The examples used here are human mesenchymal stem cells labeled for H3K4me3 and H3K27me3. For this particular example, we are monitoring adipogenic and osteogenic differentiation using the histological labeling of intracellular triglycerides and bone-specific alkaline phosphatase, respectively. When investigating other lineages, it is important to monitor differentiation using established biomarkers to ensure that the differentiation induction protocol was successful.

Materials

4% Paraformaldehyde (Electron Microscopy Sciences, 15700) (diluted with PBS)
PBS w/o Ca²⁺ or Mg²⁺ (Lonza, 17-516F)
0.1% Triton X-100 (Sigma-Aldrich, 9002-93-1) (diluted with PBS)
8% Normal Goat Serum (Cell Signaling Technology, 5425S) (diluted with PBS)
H3K4me3 ChIP grade antibody (Abcam, ab8580)
H3K27me3 ChIP grade antibody (Abcam, ab6002,)
Goat anti-Rabbit IgG Alexa Fluor 488 (Thermo-Fisher, A-11008)
Goat anti-Mouse IgG3 Alexa Fluor 594 (Thermo-Fisher, A-21155)
Fast Blue RR Salt (Sigma-Aldrich, F0500)
Naphthol AS-MX Phosphate Alkaline Solution (Sigma-Aldrich, 1596-56-1)
ProLong Gold Antifade mounting media (Thermo-Fisher, P36930)
Glass coverslips
Glass slides

Protocol Steps

Seeding and fixing cells onto glass coverslips

- 1 Following trypsinization and cell counting of desired cell type, seed cells onto glass coverslips and allow to attach for a minimum of 6 hours.
- 2 Following attachment, maintain cells in their appropriate growth/differentiation media until ready to fix and label.
- 3 Once desired timepoint is reached, wash cells gently in 1X PBS, and replace PBS with 4% Paraformaldehyde and incubate at room temperature for 20 minutes.
- 2 Remove 4% Paraformaldehyde and wash with 1X PBS, three times.

Histological labeling for alkaline phosphatase

1. Prepare diazonium salt solution by dissolving one Fast Blue RR Salt capsule (Sigma-Aldrich) in 48 ml distilled water.
2. Add 2 ml Naphthol AS-MX Phosphate Alkaline Solution and vortex.
3. Gently transfer sufficient alkaline-dye mixture to cover cells.
4. Incubate cells in staining solution at room temperature for 30 minutes protected from light.
5. Gently wash cells in deionized water three times for two minutes per wash.
6. Counter stain for nuclei with DAPI or Hoescht (optional) and wash.
7. Apply sufficient prolong gold antifade mounting media to cover cells, mount coverslip on slide

If cells were cultured in a dish, store in PBS protected from light
8. Image slides under brightfield at a low magnification (4X – 10X) to assess differentiation efficiency (Figure 3).

Histological labeling for intracellular triglycerides

1. Prepare staining solution by combining 5 ml of PBS with 140 ul of AdipoRed Assay Reagent (Lonza).
2. Gently transfer sufficient staining solution to cover cells and incubate at room temperature for 10 minutes, protected from light.
3. Wash cells in PBS three times for two minutes per wash.
4. Counter stain for nuclei with DAPI or Hoescht (optional) and wash.
5. Apply sufficient prolong gold antifade mounting media to cover cells, and mount coverslip on slide

If cells were cultured in a dish, store in PBS protected from light

6. Image slides under brightfield at a low magnification (4X – 10X) to assess differentiation efficiency (Figure 3).

Immuncytochemical labeling

1. After fixation and washing in PBS, remove PBS and permeabilize cells by incubating in 0.1% Triton X-100 for 10 minutes under gentle agitation.
2. After permeabilization, wash in PBS and then incubate in 8% Normal Goat Serum for 1 hour to block non-specific binding
3. Prepare primary antibody solution by diluting stock primary antibody in a 1:500 ratio in 8% Normal Goat Serum solution. Incubate cells overnight in primary antibody solution at 4°C under gentle agitation.
4. The next day, remove primary antibodies and wash in PBS for 30 minutes at room temperature - repeat three times.
5. Prepare secondary antibody solution by diluting stock secondary antibody in a 1:1000 ratio in 8% Normal Goat Serum solution. Incubate cells in secondary antibody solution for 2 hours at room temperature under gentle agitation, while protected from light.
6. After 2 hours, remove secondary antibody and wash in PBS for 30 minutes, four times.
7. After 2 hours of washing, mount coverslips in ProLong Gold Antifade mounting media onto glass slides.

Basic protocol 3: *High content imaging via super resolution microscopy*

This protocol describes high content image acquisition using a super resolution microscope. The example instrument used here is a Leica TCS SP8 confocal laser scanning microscope equipped with a time-gated stimulated emission depletion laser and a supercontinuum white light laser. The two example fluorophores used are Alexafluor 488 and Alexaflor 594.

Microscope (Leica G-STED) setup and operation

- 1 Properly turn on workstation and instrument.
- 2 Position a high magnification immersion objective (i.e. 100X oil immersion objective) and secure the sample. Adjust the focal plane so that the sample is focused in the ocular.
- 2 For Alexafluor 488, set supercontinuum white laser to 492 nanometer wavelength excitation, and use a hybrid detector (HyD) set to a range of 505 to 550 nanometers with a gain of 100 and offset of $-6.667E-03$, and time gate from 0.5 ns to 6.0 ns with a 499 nm gate reference wavelength.
- 3 For Alexafluor 594, set supercontinuum white laser to 592 nanometer wavelength excitation, and use a HyD set to a range of 600–655 nanometers

with a gain of 267 and offset of $-4.225\text{E-}03$, and time gate from 0.3 ns to 6.0 ns with a 499 nm gate reference wavelength.

- 4 Set z-stack heights by turning laser on continuous mode, setting the pinhole to 1 airy unit, and locating the top and bottom of the stack. Set optical section thickness to 0.5 μm .
- 5 Acquire stacks and ensure to save each section of the stack as individual images.

Basic protocol 4: *Image informatics and quantitative image analysis*

This protocol details how to process images for analysis and subsequently acquire quantitative features using ImageJ. Custom macros can be written for ImageJ, but more comprehensive scripts can be written using languages such as MATLAB or R.

- 1 Download the latest version of ImageJ software (<https://imagej.nih.gov/ij/download.html>)
- 2 Load an image stack by either selecting all of the images in the stack, and dragging them into the ImageJ window (Drag and drop feature), or importing them (File -> Import -> Image Sequence).
- 3 Compile the images into a single stack (Image -> Stacks -> Images to Stack)
- 4 Create a 2D projection of the stack (Image -> Stacks -> Z project). This will bring up a window allowing you to specify the beginning (Start slice) and end (Stop slice) of the stack, as well as the type of projection (Average Intensity, Max Intensity, Min Intensity, Sum Slices, Standard Deviation and Median). (Figure 4)
- 5 Isolate the desired signal by adjusting the threshold so that your ROIs (i.e. nuclei) are selected. (Image -> Adjust -> Threshold). Begin by adjusting the brightness (i.e. pixel intensity value) filter, and fine tune the ROIs by adjusting saturation and hue, if necessary. (Figure 5)
- 6 Select desired quantitative descriptors (Analyze -> Set measurements). Make sure to check "Limit to Threshold." (Figure 6)
- 7 To finer tune the selected threshold, specify area and circularity restrictions under analyze particles (Analyze -> Analyze Particles). To avoid partial nuclei that are cut off on the edges, check "Exclude on edges." (Figure 7)
- 8 After making the desired selections, click OK and a results window will pop up. You can save this data as an excel (.xls) spreadsheet, or you can select the data and copy it to paste in the spreadsheet application of your choice.
- 9 Open the latest version of MATLAB equipped with the Statistics and Machine Learning Toolbox (<https://www.mathworks.com/products/statistics.html>).
- 10 Download and install the MATLAB Toolbox for Dimensionality Reduction (<https://www.mathworks.com/matlabcentral/linkexchange/links/1626-the-matlab-toolbox-for-dimensionality-reduction>).

- 10 Create matrices in MATLAB to store the image analysis data, with columns corresponding to descriptors, and rows corresponding to nuclei. Group nuclei from a common condition (i.e. cell phenotype) with the same variable name.
- 11 Execute dimension reduction of the data using one of the 34 supplied techniques. For datasets containing over 10 descriptors, principal component analysis (PCA) and classical multidimensional scaling were found to work best. An example of PCA plot of H3K4me3 and H3K27me3 descriptors in basal, adipogenic and osteogenic hMSCs is shown in figure 8.

Commentary

Background Information

The abundance of functionally distinct cell phenotypes that make up a single organism are all derived from the controlled regulation of gene transcription from a common primary DNA sequence. The precise spatiotemporal orchestration of gene activation and silencing is largely governed by dynamic chromatin structures, which regulate the accessibility to specific parts of their DNA. These adjustable nucleosome architectures are highly influenced by a group of post-translational modifications (PTMs) to core histones¹, wherein specific PTMs can influence the addition or removal of other PTMs, and complex combinations of PTMs ultimately drive desired combinations of gene activation or silencing¹. Some PTMs, and the enzymes that regulate these, have been linked with cellular differentiation towards specific phenotypes², as well as cellular reprogramming to a pluripotent state^{3,4}, indicating that this “epigenetic language” is at the core of regulating specific patterns of gene transcription.

Among the numerous PTMs that have been linked to gene transcription regulation, perhaps the most widely investigated have been the co-occurring trimethylation of lysine residues 4 and 27 on histone 3 (H3K4K27me3)^{5,6}. As these bivalent marks have been shown to be associated with important transcription factors at low levels, one theory posits that their co-occurrence may serve to silence developmental genes but keep these poised for activation⁶. Desired genes can be subsequently activated or silenced by a group of lysine demethylases (KDMs) by catalyzing the removal of methyl groups from either lysine 27 (UTX & JMJD3)⁷ contributing to a chromatin structure that promotes active gene transcription, or lysine 4 (JARID1)⁸ for gene silencing.

Current methods of epigenetic analysis are mostly population based, which involve the lysis of large numbers of cells to isolate a sufficient amount of chromatin for immunoprecipitation or proteomic interrogation via mass spectrometry, both of which destroy any potential structural insight from dynamic chromatin architectures. We therefore developed image based methodologies that do not require lysing cells and fractionating histones nor isolating DNA, and can thus provide insights into how the structural organization of chromatin correlates with gene transcription regulation.

The combination of high resolution imaging and quantitative modeling has spawned the emerging field of Bioimage Informatics, which is focused on extracting and interpreting

quantitative molecular information about cellular and tissue biology and organization⁹. The ImageJ based features presented in this protocol are quickly computable and easily automatable, which allows for the fusion of high-throughput and high-content image analysis capabilities. This image analytical approach provides a unique perspective on chromatin structural dynamics and their influence on gene transcription regulation. It is relatively easy to perform and substantially reduces the time and costs required for more comprehensive methods of interrogating the epigenome, thus making them good places to begin investigations, as well as supplement data, on the structural dynamics of chromatin as a response to epigenetic modifications. With the incorporation of additional nuclear organizational data, and other developmental stem cell systems, this type of bioimage information can potentially be archived and referenced for future drug and biomaterial property screening.

Critical Parameters and Troubleshooting

There are several portions of this protocol that must be executed with precision in order to yield robust data. Particularly, the steps outlined in the *Immunocytochemical labeling* section should be strictly adhered to, as even minor modifications can lead to large differences in sample quality. The washing and blocking steps are critical to producing clean samples with minimal non-specific binding. Even small amounts of non-specifically bound fluorophores can largely affect the quantification of image features. Also, the use of a good antifade mounting medium is critical for the preservation of samples and minimization of photobleaching.

When culturing cells in preparation for labeling, it is also important to note that different biomarkers may be differentially expressed based on the cell cycle that the cell is in. Thus, when investigating such markers, one should employ a synchronization of cell cycle. One way to do this is to use an early S-phase block by applying Thymidine in two cycles to allow cells to progress synchronously through the G2 and mitotic phase.

For image analysis, it is important to prescribe the specific range of features (texture, morphology, intensity) that may be relevant to the organizational behaviors of the biomarker of choice. It is critical to note the difference between gross geometric shape descriptors, and features that are dependent on overall pixel intensity, and those that describe the relational pixel intensity to probe for spatial patterns in your image. Aside from the features that are included with ImageJ, there are a host of other descriptors that can be insightful, many of which involve processing of the image to extract specific details. For example, there are different insights that can be gained using different image filters, such as Gabor filters and Haar wavelet transforms. Patterns within an image can be analyzed using co-occurrence matrices, which quantitatively describe the relationship between neighboring pixels.

Anticipated Results

Although the examples in this protocol consisted of specific cell phenotypes and biomarkers in the context of stem cell differentiation, this approach can be applied to virtually any adherent cell type to probe for a variety of different cellular phenomena. The quality of the data ultimately depends on every step of the sample preparation, from cell culture, to

immunolabeling, to optimal image acquisition parameters set on an appropriate microscope. One should expect to be able to differentiate between different cell types and/or culture conditions based on the analysis of single cell image features. Thus, this approach is well suited to screen for drugs/small molecules or biomaterials that influence cells toward desired behaviors.

Time Considerations

The entirety of this protocol for this specific cell system, from cell culture to labeling, image acquisition and analysis, can be completed within 14–15 days. For other cell types, once the desired condition/type is achieved, the labeling/imaging/analysis can be completed within two days, based on the recommended immunolabeling steps. However, to expedite the workflow, the primary antibody incubation can be done at room temperature for 2 hours, instead of overnight at 4°C, and thus the entire process can be completed within 1 day.

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Figure 1.

Workflow overview of stem cell image analysis. Undifferentiated stem cells are induced to differentiate toward known phenotypes, after which they are immunocytochemically labeled for biomarkers of interest. High magnification/numerical aperture objectives are used in a laser scanning confocal instrument to capture high resolution images, from which image analysis software (i.e. ImageJ) can extract quantitative descriptors. These large quantitative datasets can be analyzed and interpreted using dimension reduction algorithms in MATLAB.

Figure 2.
Micrograph of hMSCs at ~50% confluency, at which point they are ready for routine passaging.

Figure 3. Histological confirmation of directed differentiation toward osteogenic and adipogenic hMSCs. Intracellular triglycerides appear red from AdipoRed staining, whereas bone-specific alkaline phosphatase stains blue. Undifferentiated hMSCs do not stain positive for either marker.

Figure 4.
2D projection of a stack in ImageJ. Depending on the biomarker type and intensity, the relevant projection type should be chosen.

Figure 5. Thresholding ROI via bandpass pixel intensity filter. With minimal background, nuclei ROIs can easily be detected via pixel intensity specification in ImageJ.

Figure 6. Menu of quantitative descriptors offered on ImageJ. Aside from Standard Deviation, Skewness and Kurtosis, the bulk of these descriptors are relevant to geometrical features of the ROIs.

Figure 7.
ROIs to be analyzed can be further thresholded by size, circularity, and position.

Figure 8.

An example PCA plot of the H3K4me3 and H3K27me3 descriptors of basal (BA), adipogenic (AD) and osteogenic (OS) hMSCs after 2 weeks of differentiation. PC = Principal Component.