A protocol for imaging spindle orientation and inheritance of fate determinants in real time.

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Method Article

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Abstract

This protocol describes a strategy to image spindle orientation during cell division in hematopoietic stem and progenitor cells in real time. This live imaging method was a modification of a method previously used to visualize the spindle using epithelial cell lines \(reference 1). This protocol also describes a strategy to track the orientation of the mitotic spindle coordinately with inheritance of the cell fate determinant Numb in real time.

Introduction

During asymmetric cell division, a dividing cell asymmetrically segregates fate determinants \(i.e. Numb) so that the daughter cells inherit different levels, thus generating daughter cells that adopt distinct cell fates. To facilitate differential inheritance of fate determinants into the two incipient daughters cells, the dividing mother cell must orient the mitotic spindle precisely along the axis of asymmetry. To determine whether a gene may be involved in this process, it may be useful to test whether gain- or loss-of-function of the gene affects spindle orientation during cell division in real time.

Reagents

• Hanks' balanced salt solution \(HBSS) \(Gibco, Life Technologies) • Fetal Bovine Serum \(Gemini Bio-Products) • FACS Antibodies: 145-2C11 \(CD3ε), GK1.5 \(CD4), 53-6.7 \(CD8), RB6-8C5 \(Ly-6G/Gr1), M1/70 \(CD11b/Mac-1), TER119 \(Ly-76/TER119), 6B2 \(CD45R/B220), MB19-1 \(CD19), 2B8 \(CD117/c-kit), D7 \(Ly-6A/E/Sca-1). All antibodies were purchased from BD Pharmigen, eBioscience or BioLegend. • RBC Lysis Buffer \(eBioscience) • X-Vivo15 \(Lonza) • 2-Mercaptoethanol \(Gibco) • Stem Cell Factor \(SCF; 100 ng/ml, R&D Systems) • Thrombopoietin \(TPO; 20 ng/ml, R&D Systems) • Iscove's modified medium-based methylcellulose medium \(Methocult M3434, StemCell Technologies). • 4-OH tamoxifen \(Sigma) • Chambered coverglass slides \(Lab-Tek II®, Thermo Scientific) • Retronectin® \(Takara Bio Inc.)

Procedure

Determining Numb inheritance in hematopoietic stem cells in real time. 1. Isolate and sort bone marrow derived hematopoietic stem cell \(HSC)-enriched cells \(c-kit+ Lin- Sca-1+; KLS) from adult mice. 2. Culture cells overnight \(~16 hrs) in X-Vivo™15 media \(Lonza) supplemented with 50 μM 2-mercaptoethanol, 10% \(vol/vol) fetal bovine serum, SCF \(100 ng/ml, R&D Systems) and thrombopoietin \(20 ng/ml, R&D Systems). 3. Infect cells with retroviruses: Numb:CFP and mCherry-α-tubulin; 48 hours after infection, harvest cells and re-sort for CFP+ mCherry+ cells. Note: Numb:CFP is specifically used since it allows clear detection of distinct levels of Numb. In contrast, we found that Numb-YFP leads to highly saturated expression of YFP and does not allow easy identification of low and high expressing daughter cells. 4. To prepare cells for imaging, plate cells in Iscove's modified medium-based methylcellulose medium \(Methocult M3434, StemCell Technologies) supplemented with 4-OH tamoxifen

\(Sigma). Note: 4-OH tamoxifen \(Sigma) was dissolved in ethanol at 1 mg/ml \(1000X), and a 1X solution was made immediately before treatment. Note: Tamoxifen is needed only if the CreER-LoxP system is being used. 5. Imaging: Using an Axio Observer.Z1 microscope with the LSM 700 scanning module \(Zeiss), collect images every 3-4 minutes with xyzt acquisition mode. Maintain cultures at 37°C, 5% CO2 using a Heating Insert P Lab-Tek S1 with an Incubator PM S1 \(Zeiss). Note: If 405 laser is used to excite Numb::CFP, identify cells with a mitotic spindle before starting setting up time-lapse imaging. This is essential since primary hematopoietic stem cells die upon prolonged exposure to this laser. 6. Determining Numb inheritance: a. Identify dividing cells in movie replay. b. Visualize cells in spectrum color format \(where red indicates pronounced α-tubulin expression and centrosome location) to readily identify the centrosomes. c. Using ImageJ software, draw a line connecting the two centrosomes of a cell \(Line 1). Subsequently, draw an additional line perpendicular to Line 1, which marks the cleavage furrow and partitions the mother cell into incipient daughter cell 1 \(D1) and daughter cell 2 \(D2). d. Using ImageJ software, determine fluorescence intensity of Numb for D1 and D2. Note: In fixed cell-based experiments, Numb was found to be ~1.8-fold higher in progentiors than in HSCs. Thus, incipient daughters that expressed at least a 1.8-fold difference in Numb expression were scored as an asymmetric Numb inheritance. Real time imaging strategy to determine spindle orientation in dividing hematopoietic stem cells. 1. Isolate and sort bone marrow derived hematopoietic stem cell \(HSC)-enriched cells \(c-kit+ Lin- Sca-1+; KLS) from adult mice. 2. Culture cells overnight \(~16 hrs) in X-Vivo™15 media \(Lonza) supplemented with 50 μM 2-mercaptoethanol, 10% \(vol/vol) fetal bovine serum, SCF \(100 ng/ml, R&D Systems) and thrombopoietin \(20 ng/ml, R&D Systems). 3. Infect cells with retroviruses: MSCV-H2B-GFP and mCherry-α-tubulin; 48 hours after infection, harvest cells and re-sort for GFP+ mCherry+ KLS cells. 4. Culture cells in 96-well U-bottomed plates \(BD Biosciences) for 48 hrs with 4-OH tamoxifen \(Sigma) Note: 4-OH tamoxifen \(Sigma) was dissolved in ethanol at 1 mg/ml \(1000X), and a 1X solution was made immediately before treatment. Note: Tamoxifen is needed only if the CreER-LoxP system is being used. 5. To prepare cells for imaging, place cells on chambered coverglass slides \(Lab-Tek II®, Thermo Scientific) coated with 0.1 μg/μl Retronectin® \(Takara Bio Inc.) in the continual presence of 4-OH tamoxifen. 6. Imaging: Using an Axio Observer.Z1 microscope with the LSM 700 scanning module \(Zeiss), collect images every 3-4 minutes with xyzt acquisition mode. Maintain cultures at 37°C, 5% CO2 using a Heating Insert P Lab-Tek S1 with an Incubator PM S1 \(Zeiss). 7. Measuring spindle orientation: a. Identify mitotic cells in movie replay. To measure spindle angle, generate a concatenation of Z-stack images of each cell at every measured time point from the start of metaphase to early telophase and display all images in orthogonally view using Zen 2010 software. b. Using ImageJ software, measure the angle formed between the substratum plane \(Retronectin base) and the virtual line passing through spindle poles.

References

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