Nuclear membrane-tethered FRAP method for measuring protein complex off-rates in live cells

Lindsey R. Pack
Stanford University https://orcid.org/0000-0002-0678-3267
Leighton H. Daigh
Stanford University
Mingyu Chung
Stanford University
Tobias Meyer (tobias1@stanford.edu)
Stanford University https://orcid.org/0000-0003-4339-3804

Method Article

Keywords: fluorescence recovery after photobleaching, live cell, cell biology, cyclin-dependent kinases, cyclins, protein-protein interactions, off-rates

Posted Date: July 8th, 2021

DOI: https://doi.org/10.21203/rs.3.pex-1479/v1

Abstract

Understanding the stability or binding affinity of protein complex members is important for understanding their regulation and roles in cells. While there are many biochemical methods to measure protein-protein interactions in vitro, these methods often rely on the ability to robustly purify components individually. Moreover, few methods have been developed to study protein complexes within live cells. Binding parameters for cyclin-dependent kinase (CDK) complexes have been challenging to measure due to difficulty expressing and purifying CDKs separately from activating cyclins. Here, we develop a method to measure off-rates of protein complex components in live-cells. Our method relies on the stable tethering of CDK to the inner nuclear membrane (Figure 1), and the utilization of FRAP to measure the off-rate of soluble, fluorescently-tagged CDK binding proteins. We use this method to study dimeric CDK complexes, measuring the off-rates of cyclins or INK4 CDK inhibitor p16 from CDKs, and trimeric CDK complexes, measuring the off-rate of cyclins and CIP/KIP CDK inhibitors p21 and p27 when bound together.

Introduction

Reagents

Cell Culture:

hTERT RPE-1 (ATCC CRL-4000)

DMEM/F12 (1:10 phenol red free) (Life Technologies 11039-047)

FBS (Sigma-Aldrich TMS-013-B)

Opti-MEM (Life Technologies 31985070

DharmaFECT 1 (Dharmacon T-2001-03)

Lipofectamine 2000 (Life Technologies 11668-019)

96 well Glass Bottom Plate with high performance #1.5 cover glass (Cellvis P96-1.5H-N)

Purified Bovine Collagen Solution, 3 mg/mL (Advanced Biomatrix 5005-B)

RPE-1 Growth Media:

500 mL of DMEM/F12 (1:10, phenol red free) is supplemented with 10% FBS and sterile filtered.

Chemicals Reagents:

Jasplakinolide (Cayman Chemical Company 11705)

Y-27632 (hydrochloride) (Cayman Chemical Company 10005583)

Latrunculin B (Abcam ab144291)

Nocodazole (Sigma-Aldrich M1404-2MG)

Plasmids:

CDK4- and CDK6-Fluorescent Protein-Δ50 lamin A in C1 backbone

Fluorescent-tagged CDK binding protein (cyclin D, p16, and/or p21/p27) in C1 backbone

Equipment

Fully automated widefield/Yokogawa spinning-disc confocal fluorescence microscope system (Intelligent Imaging Innovations, 3i) with a 60x 1.27 NA water-immersion objective. System build with Nikon Ti-E and utilizes 3i laser stack (405, 445, 488, 514, 561, 640 nm), a 3i Vector photomanipulation device, a Yokogawa CSU-W1 scanning head with dual camera port, two sCMOS cameras (Andor Zyla 4.2) enclosed by an environmental chamber (Haison), and controlled by Slidebook software (3i)

Procedure

Day 1:

1. Coat glass plates with 100 µL collagen (1:50 in PBS) for 3 hr in 37 ⁰C tissue culture incubator.
2. Remove collagen, plate 4,500-6,000 RPE-1 cells in RPE-1 growth media in each well.

Late afternoon of Day 2:

1. Prepare transfection mixtures, Opti-MEM/Plasmid and Opti-MEM/Lipofectamine, incubating separately for 5 min at room temperature. Then mix together and incubate for 30 min at room temperature.

Opti-MEM/Plasmid Mixture (volumes per well of 96 well plate):

9 µL Opti-MEM

1 µL of 1:1 CDK plasmid:CDK binding protein plasmid (0.5 µg of each plasmid)

OR

Opti-MEM/Plasmid Mixture (volumes per well of 96 well plate):

9 µL Opti-MEM

1 µL of 1:1:1 CDK plasmid:cyclin plasmid:CIP/KIP plasmid (0.5 µg of CDK plasmid, 0.25 µg of cyclin and CIP/KIP plasmid)

Opti-MEM/Lipofectamine Mixture (volumes per well of 96 well plate):

9.75 µL Opti-MEM

0.25 µL Lipofectamine 2000

2. Add 80 µL of RPE-1 growth media per well of transfection mixture prepared above.

3. Remove growth media from cells prepared the previous day, and add 100 µL of the transfection mixture per well.

4. Incubate cells with transfection mixture for 2-3 hr in 37 °C tissue culture incubator.

5. Replace transfection mixture with 100 µL of RPE-1 growth media per well.

Day 3:

1. Change media, adding 100 µL fresh RPE-1 media per well.

2. Prepare drug cocktails to prevent cell movement.

Drug Cocktail I:

247 µL RPE-1 Growth Media

3 µL 10 mM Y27631

Drug Cocktail II:

237 µL RPE-1 Growth Media

1 µL 10 mM Y27631

8 µL 1 mM Jasplakinolide

2 µL 5 mM Latrunculin B

2 µL 500 µg/mL nocodazole

3. Add 50 µL Drug Cocktail I to one well of 96 well you plan to image. Incubate 20 min at 37 □C.

4. Add 50 µL Drug Cocktail II to the same well of 96 well plate. Incubate 30 min at 37 □C.

5. While incubating with Drug Cocktail II, identify cells to image in step 6.

5a. Using widefield/Yokogawa spinning-disc confocal microscope and 60x water immersion objective described in the Equipment section, find the plane of the cell in which localization of the nuclear periphery can be observed.

5b. Use stage controls to find cells meeting the following criteria. The x,y positions of cells were saved for imaging in Step 6.

i. The cell expresses all of the constructs of interest.

ii. Sufficient expression of the CDK- Δ50 lamin A fluorescent construct that a ring of localization can be observed for the interacting protein of interest.

iii. Note that the nuclei with Δ50 lamin A expressed can form irregular contours and multi-lobed nuclei. Mild cases of these phenotypes can be used for this method.

6. Image and photobleach cells identified in step 5 for no more than 2 hr after adding drug Cocktail II.

6a. Cells were imaged every second for the first 35 seconds, followed by every 5 seconds for the next 90 seconds, and every 30 seconds until the end of the imaging. A total of 15 minutes was imaged for most conditions.

6b. Cells were photobleached after the first five seconds of imaging. Photobleaching regions were drawn as an ellipse with widths, and heights of 1 µm.

Analysis

1. In Slidebook 6.13 software, using the draw regions tools, draw the following three regions:

i. the region of interest (ROI), where the photobleaching at the nuclear lamin occurred.

ii. the reference region (REF), a distant nucleoplasmic region with the cell.

iii. the background (BG), a region absent of any cells.

2. Export the data for these three regions across the imaging time course as an excel document.

3. Generate a corrected curve for the fluorescent signal of interest using the following equation:

CORRECTED CURVE= (ROI-BG)/(REF-BG)

4. Normalize the corrected curve from 0 to 1 by subtracting the first post-photobleaching value from all time points, then dividing all time points by the average of the pre-photobleaching corrected values.

5. For a given condition, the normalized corrected curves of at least 10 cells imaged on at least two separate days of experimental setup was averaged and the standard deviation plotted.

Troubleshooting

Time Taken

Anticipated Results

References

Acknowledgements

We thank members of the Meyer lab, especially Damien Garbett and Anjali Bisaria for assistance with FRAP assays. L.R.P. was supported by National Institute of General Medical Sciences of the National Institutes of Health F32 Ruth L. Kirschstein fellowship F32GM125246. L.H.D. was supported by Stanford Medical Scientist Training Program NIH training grant T32GM007365 and National Institute of Aging of the National Institutes of Health F30 Ruth L. Kirschstein fellowship F30AG060634. T.M. was supported by a National Institute of General Medical Sciences R35 grant (GM127026).

Figures

Figure 1

Figure 1

Schematic of constructs used to localize CDKs to the nuclear periphery.