Chapter 13

Optogenetic Manipulation of Mouse Oocytes

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Abstract

Like many biological processes, oocyte development depends on careful orchestration of protein localization. Optogenetic approaches have the potential to manipulate this dynamic system with spatial and temporal precision and molecular specificity. This chapter describes the use of a photocaged chemical inducer of dimerization to control localization of genetically tagged proteins with light. As an example, we recruit a fluorescently tagged protein to one spindle pole in metaphase.

Key words Optogenetics, Dimerization, Mouse oocyte, Spindle

1 Introduction

Localizing the right protein to the right place in the cell at the right time is essential for most biological processes, and oocyte development is no exception. Experimentally manipulating protein localization on the relevant temporal and spatial scales is a powerful approach for probing such a dynamic system. Several optogenetic platforms have been successfully used in somatic cell culture systems for this purpose [1–4], and we recently reported an application in oocytes [5].

We previously developed photocaged chemical inducers of dimerization, which we used to recruit proteins from the cytosol to multiple cellular structures and to control organelle transport and kinetochore function in living cells [6–8]. Our system has three components: an anchor protein that constitutively localizes to an intracellular structure, a protein that is recruited to this structure from the cytosol by dimerization with the anchor protein, and a photocaged chemical dimerizer (Fig. 1a). The anchor protein and the recruited protein are genetically fused to the Halo tag and to *Escherichia coli* dihydrofolate reductase (eDHFR), respectively. The dimerizer is composed of a Halo ligand linked to the eDHFR ligand Trimethoprim

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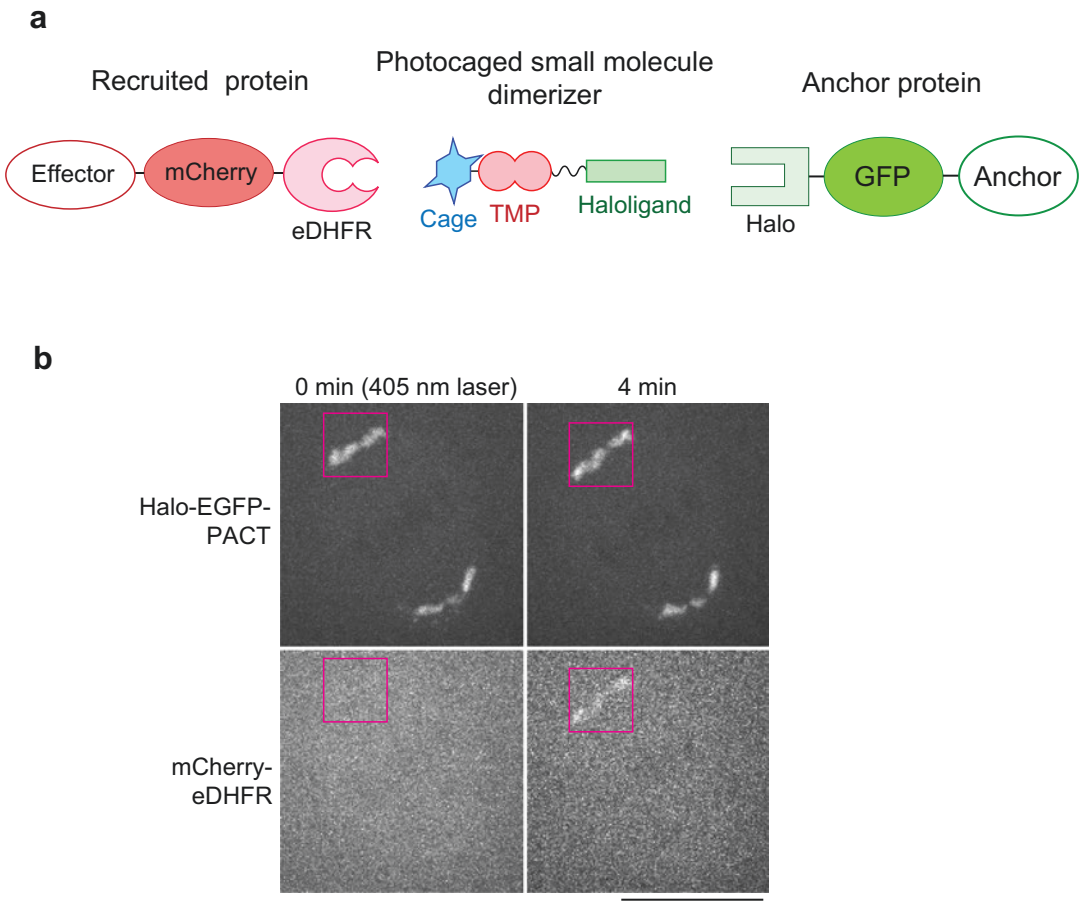

Fig. 1 Light-induced dimerization in mouse oocytes. (**a**) Schematic of light-induced dimerization. The small molecule dimerizer is composed of a Halo ligand linked to the eDHFR ligand trimethoprim (TMP), which is photocaged. An anchor protein, tagged with EGFP and Halo, localizes to a specific cellular structure, and an effector protein is tagged with mCherry and eDHFR. The dimerizer covalently binds the anchor protein at a specific structure, and the effector protein is recruited there by local uncaging with light. (**b**) Recruitment of mCherry-eDHFR to one pole of the meiosis I spindle by local uncaging (pink boxes). The anchor protein consists of a PACT domain [12], which targets to spindle poles, fused to EGFP and three tandem Halo tags. Images show before (0 min) and 4 min after uncaging

(TMP), which is photocaged. The Halo ligand covalently binds to the Halo-tagged anchor protein, and uncaging with light recruits the eDHFR-tagged protein by dimerization with the anchor protein.

In this chapter, we describe a method for light-induced dimerization in mouse oocytes in metaphase I, using recruitment to spindle poles as an example. Overall, the method involves introduction of Halo and eDHFR constructs and the chemical dimerizer to mouse oocytes and localized uncaging of the dimerizer.

2 Materials

2.1 In Vitro RNA Synthesis

1. Linearized plasmids, which harbor Halo or eDHFR constructs downstream of a T7 promoter, as templates for in vitro transcription (*see* **Note 1**).
2. T7 mScript Standard mRNA Production System (CELLSCRIPT).
3. MEGAclear Transcription Clean-Up Kit (Thermo Fisher Scientific).
4. 100% and 70% ethanol.

2.2 Oocyte Collection and Culture

1. 6–12-week-old female NSA (CF-1) mice.
2. Pregnant mare's serum gonadotropin (PMSG).
3. MEM-PVP medium [9]: measure out 0.1 g Na-pyruvate, 1 mL Gentamycin (10 mg/mL), 25 mL 1 M HEPES (pH 7.3), 3 g PVP, 1 package MEM with Earle's salts (Sigma). Add embryo culture water up to 1 L and filter-sterilize through a 0.22 mm PVDF filter. Store at 4 °C.
4. CZB medium [10]: measure out 2.385 g NaCl, 180 mg KCl, 80 mg KH_2PO_4, 145 mg $MgSO_4 \cdot 7H_2O$, 125 mg $CaCl_2 \cdot 2H_2O$, 2.11 g $NaHCO_3$, 2.215 mL Na-lactate (60% syrup), 15 mg Na-pyruvate, 20 mg EDTA-Na_2, 0.5 mL Gentamycin (10 mg/mL), 0.5 mL Phenol Red (10 mg/mL), 437.5 mg Taurine, and 1.5 g BSA. Add embryo culture water up to 500 mL and filter-sterilize through a 0.22 mm PVDF filter. Store at 4 °C.
5. 100 mM L-glutamine in water.
6. 2.5 mM milrinone in DMSO.
7. Mineral oil.
8. Dissection microscope.
9. Watch glass.
10. Tweezers.
11. 27 G needles that are fastened together.
12. Petri dishes.
13. Pasteur pipette.
14. Mouth-controlled aspiration tube for the Pasteur pipette.
15. Incubator maintaining 37 °C and 5% CO_2 in air.

2.3 mRNA Microinjection

1. Glass capillary.
2. Puller (Flaming-Brown micropipette puller).
3. Holding pipette (Eppendorf).
4. Petri dishes.
5. Inverted microscope placed on a vibration-free table.

6. Micromanipulator, TransferMan NK 2 (Eppendorf).
7. Picoinjector (Medical Systems Corp.).

2.4 Live Imaging and Light-Induced Dimerization

1. An imaging system equipped for live cell fluorescent imaging and for illuminating a subcellular region to uncage the dimerizer (*see* **Note 2**).
2. Glass-bottom tissue culture dish (FluoroDish).
3. Photocaged small molecule dimerizer, CTH [6] (stored at 1 mM in DMSO), available on request.

3 Methods

3.1 In Vitro RNA Synthesis

1. Purify linearized plasmids using the standard method of phenol/chloroform extraction followed by ethanol precipitation [11].
2. Rinse the pellet with 70% ethanol and allow pellet to dry.
3. Resuspend the pellet in RNase-free water.
4. Use 1 μg of the DNA as a template for in vitro transcription with the T7 mScript Standard mRNA Production System.
5. Purify RNA after DNase I treatment with the MEGAclear Transcription Clean-Up Kit.
6. Add posttranscriptional capping and 3′-poly(A)-tailing with the T7 mScript Standard mRNA Production System.
7. Purify mRNA with the MEGAclear Transcription Clean-Up Kit.

3.2 Oocyte Collection and Culture (See Note 3)

1. Intraperitoneally inject female mice with 5 IU PMSG 48 h before oocyte collection.
2. Prepare collection medium: MEM-PVP medium with 2.5 μM milrinone, warmed to 37 °C.
3. Prepare culture medium: CZB medium with 2.5 μM milrinone and 1 mM glutamine and allow it to equilibrate in the incubator for at least an hour.
4. Approximately 48 h after PMSG injection, dissect the ovaries and place them into a watchglass containing prewarmed collection medium.
5. Using a tweezer, anchor the ovaries to the watchglass and release the antral follicles by puncturing them several times with 27 G needles fastened together.
6. While looking through a dissection microscope, collect fully grown oocytes surrounded by cumulus cells using a mouth-controlled glass pipette.
7. Remove cumulus cells by pipetting the oocytes up and down with a smaller diameter pipette.

3.3 mRNA Microinjection

1. Prepare injection medium: collection medium with 0.3% BSA.
2. Make injection pipette by pulling a glass capillary with a Flaming-Brown micropipette puller (Model P-97) with the following settings: *P* = 500, Heat = 300, Pull = 150, Vel = 100, Time = 150.
3. Place the holding and injection pipettes into capillary holders on the motor module of the micromanipulator.
4. Prepare the microinjection dish by placing a 160 μL oval-shape drop of microinjection medium close to a 0.3 μL mRNA drop (*see* **Note 4**). Cover with mineral oil and line up oocytes in the microinjection medium for sequentially microinjection.
5. Place the microinjection dish on the microscope stage and position the holding and injection pipettes into the drop of microinjection medium.
6. Open the tip of the injection pipette by gently tapping it against the holding pipette.
7. Setup the picoinjector to PBal = 2 psi, PInj = 7.5 psi, PClear = 12 psi, time = 3 s. This results in an injection volume of 5–10 pl.
8. Fill the injection pipette with mRNA solution.
9. Capture an oocyte using the holding pipette and align the injection pipette, oocyte, and holding pipette along one axis.
10. Pierce the plasma membrane with the injection pipette and press INJECT. Avoid the nucleus.
11. Withdraw the injection pipette. Release the oocyte from the holding pipette and repeat.
12. Once all oocytes are injected, transfer them to the culture dish with culture medium covered with mineral oil.
13. Hold in the incubator overnight to allow protein expression.

3.4 Live Imaging and Light-Induced Dimerization

1. Prepare maturation medium: CZB medium with 1 mM glutamine, and allow it to equilibrate in the incubator for at least an hour.
2. To allow meiotic resumption, washout milrinone by transferring oocytes through 5 drops of 100 μL maturation medium and transfer to maturation medium covered with mineral oil in a petri dish.
3. Warm up the environmental chamber (37 °C) and turn on the CO_2 regulator (5% in air).
4. Prepare a petri dish with a 100 μL drop of maturation medium +1 μM CTH (*see* **Note 5**).
5. Four hours after milrinone washout, transfer oocytes to the dish with maturation medium + CTH and incubate for an hour.

6. Prepare a glass-bottom tissue culture dish with several 3 μL drops of maturation medium drops covered with mineral oil for live imaging dish.
7. Washout CTH by transferring oocytes through three drops of 100 μL maturation medium, then transfer oocytes to the imaging dish and further incubate for an hour.
8. Place the imaging dish on the microscope stage and look for oocytes that are expressing both Halo and eDHFR constructs based on the fluorescent tags.
9. Uncage CTH in a selected region. Our example (Fig. 1b) shows uncaging at one spindle pole using a targeted 405 nm laser (*see* **Notes 6** and **7**).
10. Follow the cell by live imaging to confirm the recruitment of eDHFR constructs. Cells can also be fixed and stained to document the effects of recruitment.

4 Notes

1. When designing a construct that fuses eDHFR to your effector of interest (e.g., kinases, motor proteins), it is preferable to remove the domain that is important for its endogenous localization. This will keep the eDHFR fusion protein freely diffusing in the cytosol until recruitment. Here we use 3Halo-EGFP-PACT as the anchor protein for targeting to spindle poles and mCherry-eDHFR as the recruited protein, but any effector of interest can be fused to this construct.
2. Our imaging system consists of a microscope (DMI4000 B; Leica) equipped with a 63 × 1.3 NA glycerol-immersion objective lens, an xy piezo Z stage (Applied Scientific Instrumentation), a spinning disk confocal scanner (Yokogawa Corporation of America), an electron multiplier charge-coupled device camera (ImageEM C9100-13; Hamamatsu Photonics), and an LMM5 laser merge module with 488- and 593-nm diode lasers (Spectral Applied Research) controlled by MetaMorph software (Molecular Devices). We use a heated environmental chamber with a stage top incubator (Incubator BL and Heating Insert P; PeCon GmBH) to maintain 37 °C and 5% CO_2 in air. For local uncaging we use an iLas2 illuminator system (Roper Scientific), equipped with a 405 nm laser (CrystaLaser LC model # DL405-050-O) with a maximum output of 27 mW after fiber coupling, controlled using the iLas2 software module within MetaMorph.
3. This procedure is described in detail with video in reference [9].

4. In this example we use 3Halo-EGFP-PACT at 1500 ng/μL and mCherry-eDHFR at 10 ng/μL. It is critical to keep the concentration of the recruited protein very low. With low cytosolic levels, it is easier to detect recruitment and keep global activity of the effector low until it is enriched locally by recruitment.
5. To minimize premature uncaging of CTH, avoid light exposure by keeping it in the dark as much as possible or using a red light.
6. Vary the intensity of the targeted laser to optimize recruitment while minimizing photobleaching and phototoxicity.
7. In general, recruitment happens in a minute, and the duration of the recruitment depends on the turnover rate of the anchor protein. With 3Halo-EGFP-PACT for example, spindle pole recruitment lasts for at least 60 min.

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