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Kaede-Centrin1 labeling of mother and daughter centrosomes in mammalian neocortical neural progenitors

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Abstract

The importance of the centrosome in regulating basic cellular processes and cell fate decisions has become increasingly evident from recent studies tracing the etiology of developmental disorders to mutations in genes encoding centrosomal proteins (Nigg and Raff, 2009). This unit details a protocol for a fluorescence-based pulse-labeling of centrioles of neural progenitor cells in the developing neocortex of mice. *In utero* electroporation of Kaede-Centrin1 followed by *in utero* or *ex vivo* photoconversion allows a direct monitoring of the inheritance of centrosomes containing centrioles of different ages in dividing neocortical neural progenitors (*i.e.*, radial glial cells). This is achieved by combining the irreversible photoconversion capacity of Kaede protein from green to red fluorescence with the faithful duplication of the centrosome during each cell cycle. After two mitotic divisions following photoconversion, mother centrosomes containing the original labeled centriole appear in both red and green fluorescence, and can be distinguished from daughter centrosomes which appear in green fluorescence only. This facilitates the study of the inheritance and behavior of the mother and daughter centrosomes in asymmetric cell divisions in the developing mammalian neocortex.

Keywords

centrosome; Kaede-Centrin1; mother and daughter centrosomes; photoconversion; neocortex; radial glia progenitor cell; *in utero* electroporation

Introduction

This unit details a protocol for labeling the centrioles of neural progenitor cells (*i.e.*, radial glia) in the developing neocortex of mice during the peak period of cortical neurogenesis (gestational days 13.5 through 17.5, *i.e.*, E13.5-17.5), so as to study centrosome segregation in the context of neurogenesis (Wang *et al.*, 2009). Centrioles/centrosomes of radial glia are initially labeled in green fluorescence by *in utero* electroporation of a plasmid bearing the photoconvertible fluorescent protein (Kaede) fused to the centriolar protein Centrin1 (Kaede-Centrin1) into the developing neocortex of embryos at E13.5. The plasmid is taken up by the radial glia within the ventricular zone. Approximately 24 hours later, each electroporated embryo is exposed to a brief pulse of violet light while in the uterus to convert the Kaede-Centrin1 protein from green to red fluorescence. The uterus is placed back into the mouse, and the embryos are allowed to continue development. About 48 hours later, the brains of the embryos are recovered and two distinct populations of centrosomes can be observed: one has both green and red fluorescence, representing the more mature mother centrosomes, and the other has green fluorescence only, representing the less mature daughter centrosomes. We have shown that mother centrosomes are preferentially inherited by the renewing radial glia remaining in the ventricular zone, while daughter centrosomes

are mostly inherited by the differentiating progeny that migrate away from the ventricular zone and occupy more dorsal layers of the neocortex, including the cortical plate (Wang *et al.*, 2009). These results suggest that centrosome inheritance is tightly regulated and coordinated with cell fate decisions during asymmetric division of neural progenitors in the developing neocortex.

This unit first describes the well-established method of *in utero* electroporation of plasmid DNA into radial glia in the developing neocortex (Basic Protocol 1). Next, a method for photoconverting Kaede-Centrin1 *in vivo* is described (Basic Protocol 2), followed by a procedure for preserving and visualizing the centrosomes with different fluorescence spectra. Finally, an alternative protocol to photoconvert Kaede-Centrin1 in organotypic neocortical slices for time-lapse imaging studies of centrosome regulation during neurogenesis is presented (Basic Protocol 3).

NOTE: This protocol was developed in mice and therefore some parameters must be determined empirically when applied to other species.

NOTE: All solutions and equipment contacting the embryos should be sterile and the procedure should be approved by the Institutional Animal Care and Use Committee (IACUC) of individual institutions.

Basic Protocol 1: *In utero* electroporation

This protocol has been previously described (*e.g.*, Tabata and Nakajima, 2008) and extensively employed in studying the function of gene(s) of interest in mammalian neocortical development.

Materials

- Electroporation system (BTX, ECM830, Harvard Apparatus)
- Isofluorane dispenser (VetEquip)
- Silk or nylon sutures
- Wound clips and clipper (7mm; CellPoint Scientific Inc.)
- 10-ml sterile syringe
- Sterile PBS and penicillin (100 I.U./ml)/streptomycin (100 mg/ml) in PBS, warmed to 37°C
- Heating pad and disposable underpads
- Sterile gauze
- Ethanol and iodine wipes
- Glass capillary injection needles (tip diameter ~100 µm; beveled)
- Plasmid DNA (Kaede-Centrin1, 3.0 µg/µl) mixed with Fast Green dye (Fisher Biotech) (1% in PBS, 1 µl dye per 10 µl DNA solution)

NOTE: The use of endotoxin-free plasmid DNA (*e.g.*, prepared using Qiagen Endotoxin-free Maxiprep kit) is recommended.

1. A timed pregnant female mouse at 13.5 days of gestation (*i.e.*, embryonic day 13.5 or E13.5) is anesthetized with isofluorane. The mouse is transferred from the isofluorane induction chamber to the surgery table onto a heating pad (37-40°C) covered with a clean disposable underpad, with its head placed in a nose cone

connected to the isoflurane output tube. The flow rate of the isoflurane is as per the dispenser manufacturer's directions (e.g., 1-4 L/min. to the chamber and 0.5 L/minute to the nose cone).

2. When the mouse is unresponsive to toe pinches, the abdominal fur is removed with a hair clipper. The shaven skin is cleaned with iodine and alcohol wipes and a vertical incision (~2.5 cm) is made to the skin. To facilitate subsequent suturing of the muscle, the skin is separated from the underlying muscle by cutting away the connective tissue. A slightly smaller incision is made to the muscle (Fig.1A).

NOTE: From this point forth, the exposed abdominal tissue as well as the embryos should be continually moistened with warm (37°C) PBS dispensed dropwise from a 10-ml syringe.

3. A sterile gauze with a central hole cut out is placed upon the abdomen such that only the incision area is exposed. The uterine horns are gently pulled out of the abdominal cavity and placed on the gauze (Fig. 1B).
4. At E13.5, the lateral ventricles of the developing brain occupy a large portion of each brain hemisphere and can be discerned through the uterine wall and yolk sac as a slightly darker, crescent-shaped area. The embryos are gently rotated within their yolk sacs with the aid of a spatula so as to position the head at an optimal angle for injection (Fig. 1C), with care taken not to squeeze the embryos. Plasmid DNA-dye mixture (~1 µl) is injected into the lateral ventricle of each embryo using a beveled glass micropipette (Fig. 1D). As the DNA-dye mixture fills the ventricle, the ventricle will become more visible as a green crescent shape.

NOTE: In order to efficiently perform the photoconversion (Basic Protocol 2) the following day and minimize surgery time, it is advisable to make the injection on the same side for each embryo.

5. After injection, the embryo is pulsed with a train of five 40-50 mV pulses (duration: 50 msec; interval: 950 msec) by covering the injected area with the positive electrode while the negative electrode maintains contact with the embryo on the diametrically opposite side of the head or body (Fig. 1E). Radial glia line the ventricle in the area known as the ventricular zone, and therefore take up the plasmid DNA when the voltage pulses are applied. As development proceeds, radial glia divide asymmetrically to produce radial glia that remain in the ventricular zone, as well as more fate-restricted daughter cells that migrate radially away from the ventricular zone to occupy more dorsal layers of the cortex.

NOTE: Care should be taken to avoid contact between the electrodes and the placenta during pulse application.

6. The uterine horns are placed back into the abdominal cavity and bathed in antibiotic-PBS solution. The wound is sutured and clipped (Figs. 1F and 1G). Analgesic ointment is applied with a cotton-tipped applicator to the wound area and the mouse is placed separately in a clean cage and closely monitored for respiratory distress until it is alert and ambulatory.

Basic Protocol 2: *In utero* photoconversion

Before undertaking this protocol, the minimum time required for complete photoconversion of Kaede-Centrin1 from green to red fluorescence should be tested. For this calibration procedure, embryos are injected at E13.5, and the following day (E14.5) the embryos are exposed to violet light (see below); around 3 to 5 minutes is usually sufficient. The light beam will penetrate the uterine wall, the yolk sac and outer tissues of the embryo's head to

the deepest layer of the developing neocortex, *i.e.*, the ventricular zone where the Kaede-Centrin1-expressing radial glia are located. Embryos can be sacrificed immediately following the photoconversion in these pilot experiments to examine the efficacy of the photoconversion.

Additional materials

- Source of violet light (*e.g.*, a fluorescence dissection microscope with a mercury burner and a 4',6-diamidino-2-phenylindole (DAPI) filter)
 - Wound clip remover
1. Approximately 24 hours after *in utero* electroporation, the mouse is anesthetized with isoflurane (see Protocol 1) and the wound is re-opened by removing wound clips and sutures. The uterine horns are exposed and each embryo is held under the beam of a violet light (350-400nm), which is focused on the injected area, for 3-5 minutes (Fig. 2).
 2. After all embryos are treated, the uterine horns are placed back into the abdominal cavity and the wound is re-sutured and clipped. Appropriate analgesia is applied and the mouse is closely monitored until it has recovered, as described in Basic Protocol 1.

Tissue preservation, imaging and analysis

Materials

- 4% paraformaldehyde (PFA), freshly prepared (cold)
 - 1× phosphate-buffered saline (PBS), pH 7.4 (cold)
 - 4% Avertin (2, 2, 2-Tribromoethanol; Sigma) in PBS
 - 1-ml syringe with 30G1 size needle for Avertin injection
 - Two 10-ml syringes connected by a stopcock, with 30G1/2 size needle attached to tubing (Fig. 3A)
 - Dissection dish (60 mm Pyrex dish lined with 7-8 mm of Sylgard 184 Silicone Elastomer)
 - Insect pins (Fine Scientific Tools)
 - Dissection tools for removing brain (fine forceps, scissors, spatula)
1. 48 hours after photoconversion, the mouse is sacrificed by intraperitoneal injection of a lethal dose of Avertin (~0.8 ml). When the animal is unresponsive to toe pinches but still breathing, the abdominal cavity is cut open and the uterine horns exposed.
 2. Each embryo is removed from its yolk sac (Fig. 3B), pinned to a dissection dish (Fig. 3C) under a dissecting microscope and transcidentally perfused with cold PBS (pH 7.4) followed by freshly prepared cold 4% PFA in PBS. The perfusion needle is inserted into the left ventricle of the beating heart, and a small incision is made to the right atrium to permit the outflow of blood (Fig. 3D). Cold PBS is slowly dispensed from the syringe (3-4 ml is usually sufficient for E16.5 embryos) until the outflow of blood from the right atrium has ceased and the embryo is white (Fig. 3E). The stopcock is adjusted to the open position for cold PFA and a similar volume of PFA is infused through the heart.

3. The brain is dissected (Fig. 3F & G) and post-fixed overnight at 4°C with 4% PFA in PBS. The following day, the brain is rinsed with PBS, embedded in 3-4% agarose and sectioned at 50-80 µm in the coronal plane using a vibratome (Leica Microsystems).
4. Floating sections are collected in a 48-well dish (each well containing PBS with 0.03% sodium azide). Centrosomes can be imaged by confocal laser scanning microscopy using a 40× or 60× objective (Olympus FV1000). Alternatively, sections can be mounted onto glass slides and covered with mounting medium and coverslip for imaging.

Basic Protocol 3: *Ex vivo* photoconversion in brain slices and time-lapse imaging

The same principles used for *in vivo* photoconversion can be applied to organotypic brain slice cultures, when the desired goal is a time-lapse imaging study of mother and daughter centrosome behavior over the course of neocortical neurogenesis. As for Protocol 2, the minimum time required for complete photoconversion should be determined for slices in pilot experiments. Usually a much shorter time (less than 1 minute) is required to achieve near-complete photoconversion. The protocol for preparing organotypic neocortical slice cultures has been previously described (Elias and Kriegstein, 2007; Daza *et al.*, 2007).

1. *In utero* electroporation of Kaede-Centrin1 at E13.5 is performed as described above (Basic Protocol 1).
2. One day later, the brain is dissected from the live embryo and embedded in 4% agarose in artificial cerebrospinal fluid (ACSF).
3. Coronal sections of the neocortex (~300-400 µm) are prepared using a vibratome (Leica Microsystems) and placed onto membrane inserts (Millicell, Millipore) in a 35-cm glass-bottom Petri dish with 750 µl of slice culture medium. The cultures are maintained in a humidified incubator (37 °C and 5% CO₂) for days.
4. For photoconversion, the dish containing the organotypic culture slices is transferred to an inverted microscope (*e.g.*, Axiovert 200, Zeiss) with a mercury burner and a DAPI filter. The slices are exposed to a brief (*i.e.*, hundreds of milliseconds to a few seconds) pulse of epifluorescent illumination using a DAPI filter.
5. The slices are returned to the incubator and images of labeled centrosomes are taken at the desired time point. The clear segregation of mother and daughter centrosomes can be witnessed after 1.5 to 2 divisions of the radial glia, *i.e.*, approximately 48 hrs after photoconversion.

Materials

Slice culture insert (Millicell, Millipore)

Glass-bottom Petri dish (MatTek Corporation)

Reagents and Solutions

Artificial cerebrospinal fluid (ACSF; pH 7.4, 310 mOsm l⁻¹)

125 mM NaCl

5 mM KCl

1.25 mM NaH₂PO₄

1 mM MgSO₄
 2 mM CaCl₂
 25 mM NaHCO₃
 20 mM glucose

Brain slice culture medium

50% BME
 25% Hanks
 5% FBS
 1% N-2
 1% penicillin/streptomycin/glutamine (Invitrogen/GIBCO)
 0.66% D-(+)-glucose (Sigma)

Commentary

Background information

The discovery and characterization of many novel centrosomal proteins in recent years has shed light on the varied and essential functions of the centrosome in controlling cell behaviors and cell fate decisions during development (Schatten and Sun, 2010). These functions may be especially important in a complex tissue such as the mammalian neocortex, a multi-layer structure whose organization depends upon a tightly regulated balance between progenitor cell self-renewal and differentiation. The neocortex begins as a layer of neuroepithelial cells surrounding the lateral ventricles at around E9.5 in mice (Molyneaux *et al.*, 2007). Neuroepithelial cells divide to give rise to radial glia, a major population of neural progenitors in the developing neocortex that undergo asymmetric division to self-renew and also to produce more fate-restricted cell types, including neurons (Götz & Huttner, 2005). The precise mechanisms that direct the asymmetric division of radial glia are not entirely clear; however, the differential inheritance of mother and daughter centrosomes by radial glia and their more differentiated daughter cells has recently been observed in the developing brain, and this segregation has been shown to be critical for maintaining the pool of neural progenitors (Wang *et al.*, 2009). The asymmetric segregation of mother versus daughter centrosomes has been previously reported in other organisms such as *Drosophila* (for review see Yamashita & Fuller (2008)), suggesting that the regulation of centrosome inheritance may be a conserved mechanism for ensuring the proper balance between progenitor maintenance and differentiation during asymmetric cell division in eukaryotes. The Kaede-Centrin1 pulse-labeling and photoconversion protocol distinguishes between mother and daughter centrosomes, allowing a closer study of this mechanism in real-time in the developing mammalian brain (Fig. 4).

Critical parameters and troubleshooting

The combination of *in utero* electroporation followed by *in utero* photoconversion is stressful for the mouse and can result in a high rate of embryo mortality. There are a few key points to heed when performing these procedures that can increase the probability of embryo survival. In general, the surgeries for *in utero* injection and electroporation and photoconversion should be performed in the minimum amount of time allowed by the experimenter's skills. The embryos, as well as the mother's internal organs and overlying abdominal muscle and skin, should be kept moist (with warm PBS) at all times. For Basic Protocol 2, the time of exposure to violet light is the critical parameter. If the time is too

short, effective photoconversion cannot take place; however, too long an exposure of the embryos to the outer environment can substantially decrease viability. It is worth noting that with repeated use, the intensity of light from a mercury burner decreases. If possible, a bulb dedicated to photoconversion should be set aside to be used only for this purpose so as to keep track of the bulb use time. In addition, to maximize photoconversion the embryos should be positioned under the light beam such that the beam covers the entire surface of the cortex on the injected side of the brain. Finally, Kaede is a highly photo-labile protein that is easily converted from green to red fluorescent by prolonged exposure to ambient levels of UV light or a brief exposure to (unfiltered) white light from a mercury burner (Fig. 5). This is true even for fixed tissue. Thus, care must be taken during processing and handling of the tissue and subsequent imaging in order to protect the tissue from any unwanted photoconversion.

Time considerations and anticipated results

Centrosome duplication is initiated at the G₁/S transition and completed before mitosis, and an additional 1.5 to 2 cell divisions are required for the nascent centriole to fully mature (Anderson and Stearns, 2009; Delattre and Gonczy, 2004). During the peak phase of neurogenesis in mice (E13.5-16.5), the cell cycle length is approximately 12 to 18 hours (Mitsuhashi and Takahashi, 2009). Therefore the segregation of mother centrosome- and daughter centrosome-containing cells can be observed in the period of about 36 to 48 hours after photoconversion. If a complete photoconversion of Kaede protein takes place, one expects that green and red fluorescent centrosomes (*i.e.*, mother centrosomes) remain primarily at the ventricular zone surface where radial glia reside, while centrosomes with green fluorescence only (*i.e.*, daughter centrosomes) populate the more dorsal layers of the neocortex where the differentiated progeny of radial glia have migrated (Fig. 6).

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Figure 1.

Procedure for *in utero* electroporation. (A) Timed pregnant female mouse at gestational/embryonic day 13.5 (E13.5) under isofluorane anesthesia with abdomen shaven and cleaned. The skin and underlying muscle have been cut open. (B) Mouse covered with a square of sterile gauze with a central hole cut out to expose only the opened abdominal cavity. The uterine horn containing embryos on the right side of the mouse has been gently lifted out of the cavity and placed on the gauze. (C) Positioning the embryo for injection by using a spatula to gently roll the embryo around within the yolk sac. (D) Glass capillary micropipette filled with plasmid DNA-dye mixture penetrating the lateral ventricle of the embryo through the uterine wall and yolk sac. (E) Positioning the electrodes for pulse delivery with the positive electrode covering injected area and the negative electrode contacting the embryo at a diametrically opposed location. (F) Suturing the abdominal muscle after placing the uterine horn back into the abdominal cavity. (G) Applying wound clips to the skin.

Figure 2.
Set-up for *in utero* photoconversion. The embryo is held under a beam of violet light to effect photoconversion. The beam of light covers the injected area.

Figure 3.

Set-up and procedure for embryo dissection, transcatheter perfusion and brain dissection. (A) Syringes containing cold phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) connected by a two-way stopcock with attached tubing and needle for transcatheter perfusion. (B) Dissection of embryo (gestational day 16.5) from uterus of anesthetized mouse. (C-E, ventral view) (C) Dissected embryo pinned facing upward to a dissection dish filled with cold PBS. (D) Close-up view of exposed but intact heart with arrows to indicate the flow of perfusion solutions into the left ventricle (LV) and out of the right atrium (RA). (E) Embryo after perfusion; heart chambers are outlined by a broken white line. Note the white color of the head when perfusion is complete. LA, left atrium; RV, right ventricle. (F-G, dorsal view) (F) Perfused embryo pinned facing downward, with cranial skin and bone being removed with fine forceps. (G) Exposed brain being removed with spatula. The left (LH) and right (RH) hemispheres of the brain are outlined by a broken white line.

Figure 4.

Kaede-Centrin1 labeling of centrosome. Kaede-Centrin1-labeled centrosome imaged two days after photoconversion with mother centriole (red), daughter centriole (green) and stained for a centrosomal protein, γ -tubulin (blue). The overlay image is shown on the right. Scale bar = 2 μ m.

Figure 5.

Fluorescent properties of Kaede-Centrin1 before and after photoconversion (PC). Plasmid DNA encoding Kaede-Centrin1 was injected into the lateral ventricle of an embryo at gestational day 13.5 (E13.5) and the embryo was perfused the following day (E14.5). A 60- μ m floating brain section was first imaged using a confocal microscope in green (488) and red (546) channels. Kaede-Centrin1 expression is detected in the ventricular zone (VZ) and subventricular zone (SVZ) in the green channel only. Following a 15-sec. exposure to unfiltered white light from a mercury lamp, the section was imaged a second time with the same laser output and detection parameters. Note the complete conversion of Kaede-Centrin1 from green- to red-fluorescent. Scale bar = 10 μ m.

Figure 6. Segregation of mother and daughter centrosomes in the developing neocortex after pulse-labeling with Kaede-Centrin1. Embryonic neocortex (NCX) at gestational day 16.5 (E16.5) treated with a nuclear stain. The ganglionic eminence (GE) and pial surface (Pia) are indicated as points of reference. Scale bar = 200 μ m. Plasmid DNA expressing Kaede-Centrin1 was injected into the lateral ventricle of an embryo at E13.5, and the embryo was exposed *in utero* to violet light at E14.5 to effect photoconversion of the Kaede-Centrin1 protein from green to red. The embryo was perfused at E16.5 and a 60- μ m floating brain section was imaged by confocal microscopy. Mother centrosomes retain red fluorescence from the photoconversion, and are primarily localized in the ventricular zone (VZ), the cortical progenitor niche (2). Daughter centrosomes, born after the pulse of violet light was administered, are green only and are found in both the VZ as well as in the cortical plate (1), where differentiated neurons have migrated. Note the absence of red fluorescence signal in this area. Scale bar = 10 μ m.