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AGAR-OVERLAY TECHNIQUE FOR *DICTYOSTELIUM*

(Fukui Last Revision: June, 1998)

(Note) This protocol was originally developed in 1984 for the immunofluorescence staining with Dr. Shigehiko Yumura, then my graduate student at Department of Biology, Osaka University (ref. 1, 2). The technical detail is described in ref. 3, 4. More recently, we have applied this technique to apply to live cell observations (ref. 5, 6).

(References)

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2. Yumura, S., and Fukui, Y. (1985). Reversible cyclic AMP-dependent change in distribution of myosin thick filaments in *Dictyostelium*. *Nature* **314**, 194-196.
3. Fukui, Y. et al. (1986). High-resolution immunofluorescence for the study of the contractile apparatus. In "The Contractile Apparatus and the Ctyoskeleton" ("Structure and Contractile Proteins", *Methods in Enzymology**134***, Pard D), ed R. B. Vallee, Academic Press, pp. 573-580.
4. Fukui, Y. et al. (1987). Agar-overlay immunofluorescence: high resolution studies of cytoskeletal components and their changes during chemotaxis. In "*Dictyostelium discoideum*: Molecular Approaches to Cell Biology", *Methods in Cell Biology* **28**, ed. J. A. Spudich, Academic Press, pp. 573-580.
5. Fukui, Y., and Inoué, S. (1991). Cell division in *Dictyostelium* with special emphasis on actomyosin organization in cytokinesis. *Cell Motil. and Cytoskel*. **18**, 41-54.
6. Fukui, Y., and S. Inoué (1997). Amoeboid movement anchored by eupodia, new actin-rich knobby feet in *Dictyostelium. Cell Motility and Cytoskeleton*. **36**, 339-354.

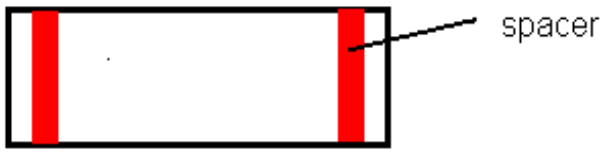
[A] Preparation of Agarose Sheet

1. Dissolve 1 g of **agarose-M** (LKB Pharmacia: Cat. #2206-101) in 50 ml of buffer to make a 2% (w/v) solution. I use 17 mM Na/K-phospahte buffer (pH 6.5), or Bonner's salt solution (10 mM KCl, 10 mM NaCl, 3 mM $CaCl_2$) for *Dictyostelium* cells. Autoclave for 15 minutes, and keep it in a refrigerator for a long time storage, up to 12 months or longer. Every time you use the agarose, re-dissolve the agarose in a hot water bath, and maintain the temperature of the bath as high as possible. I do not recommend to use a classic microwave oven because it often boil and burn agaose (a new type of “intelligent” microwave oven probably works but I have not tested yet.)

2. Detergent clean high quality slide glasses, and wipe with ethanol immediately before the preparation. Using a fine glass cutter with a diamond tip, cut a

Corning 22 x 22 mm, No. 1-½ coverslip (cat. #2870) into strips about 3 mm wide. These strips are 0.19 mm thick and serve as the spacers.

3. Place a piece of clean slide glass on a flat bench, ideally black colored, and align the two spacers on both edges of the slide using forceps. The black-top bench makes it easy to view the thin agarose.



4. Drop about 1 ml of hot agarose on the slide, and overlay with the second slide glass on top of the agarose drop. Gently but quickly hold down the slide glass to spread the agarose before it gels. Keep holding the upper slide at the position on top of the spacers until the agarose gels. It will gel in 15 seconds.

5. Using a pair of razor blades, remove excess agarose from edges of the slide-agarose-slide "sandwich". Store this sandwich in a Petri dish containing about 20 ml of the buffer, and keep it in a refrigerator. We can store as long as a month or until you notice fungi growing. I seal the Petri dish with Parafilm.

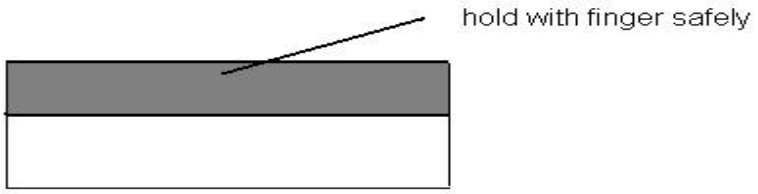
[B] Preparation of the Agar-overlay Sample

1. Carefully remove the upper slide glass from the sandwich. We can use a pair of forceps to handle the slide.

2. Place two layers of Whatman no. 1 filter paper in a Petri dish, and saturate with distilled water. This serves as an incubation chamber maintainign 100% humidity. Place a slide glass on the filter paper, and then a Corning no. 1 coverslip (cat. no. 2865) on top of the slide glass. We should not try to clearn this coverslip (use as it is). The slide glass is just for a support of the coverslip. Drop a small droplet of water before you place the coverslip onto the slide glass so that the surface tension will fix the coverslip in place.

3. Drop an aliquot of cell suspension (about 25 µl) on the coverslip, and incubate for 2-3 minutes to let the floating cells attach to the coverslip. Remove excess buffer from the coverslip, but you have to leave a touch of , resonable amount (~5 µl) of the buffer on the coverslip.

4. Using a pair of sharp stainless razor blades (ex., GEM Super Stainless Razor; we can buy at Walgreens), cut the agarose sheet into 7-8 mm square pieces. With the razor, pick up one piece of the agarose, and drape over the cell suspension on the coverslip.



5. Remove excess buffer from the agar-overlaid sample. This is a critical step. First, using a Pasteur pipet, then using a small strip of Whatman no. 1 filter paer. Remove most of the buffer from the edges and surface. We can touch the filter paper gently at the edges of the agarose to blot off the buffer. I usually hold the sample with about 45 ° angle to let the buffer drop to the lower edge of the agarose, and blot it off from the corner. Inspect the cells under a phase-contrast microscope (20x objective). For a long term incubation, examine the cells every hour or so, and if the cells are too thin, supply 0.5-1 µl of the buffer with a fine micro-tip pipette. Efforts to provide the best welfare to cells are crucial for good results.

[C] Fixation and Staining

(Note) Fixation requires a protocol optimal to your cells, structures ,and antigens: so you are assumed to have already tested and established a good fixation protocol for your system.

1. For myosin staining (in *Dictyostelium*):
Mix 27 ml of concentrated (37%) formalin (ex. Mallinckrodt cat. #5016) with 973 ml of absolute methanol (ex. Baxter, anhydrous, cat. #4324), pour into 500 ml glass bottle, and add generous amount of hygroscopic granules (Molecular Sieves) (Aldrich, cat. # 20,858-2; Fisher, cat. # M-564). Store in a deep-freezer (-20°C). You can store at least 6 months.

2. Pour the fixative into a plastic beaker, about 300 ml capacity, and place a pre-cooled porcelain staining rack (“Coors”: sold by Thomas Scientific) in the beaker. Measure the temperature of the fixative with a thermometer.

If it is colder than -15°C, warm up to between -13 to -15°C. Never fix the agar-overlaid sample with the fixative colder than -15°C, that causes a total disruption of the structure due to freezing, particularly for fixation by acetone, rather than methanol.

3. In a single, quick motion, dip the sample into the fixative and place on the staining rack. Fix for 4 to 5 minutes in the deep-freezer (-20 °C).

4. After bringing the beaker to the lab bench, all the following steps are done at the room temperature. Take the staining rack out of the fixative, and let the sample partially dry in the air (this prevent the agarose coming off). Wash the sample three times, 5 minutes each, with PBS in the plastic beaker. In the second wash, remove the agarose from the sample by gentry peeling off one corner of the agarose in PBS. I use a hand-made fine needle attached to a glass rod.

5. After washing, process the staining by your own standard immuno-fluorescence staining method.

(Mounting medium)

Vinol-205 (polyvinyl alcohol)
Air Products & Chemicals, Inc. (1-800-523-9374)

Vinol	25 g
PBS	100 ml
Glycerol	50 ml

Mix on a heavy duty stirrer at the room temperature for one day. Remove precipitate, if any, by centrifugation (13,500 rpm, 30 min). Store at the room temperature. Will last for years.

(Anti-quenching agent)

DABCO (Aldrich; Cat. # D2,780-2)
Add about 5% (v/v) crystal, mix by passing through a Pasteur pipet until the crystal dissolves. Spind down at 13,000 rpm for 5 min to remove air bubbles.

Good Luck!