

Microinjection Technique for *Dictyostelium*

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(Note) This chapter is the updated technical note intended for Dictyostelium researchers. The original note has been published in “CELL BIOLOGY: A Laboratory Handbook” (1998).

I. Introduction

Introducing molecules into live cells is best applied to **fluorescent analogue cytochemistry** (Taylor and Wang, 1978). This technique has been used for studying cytoskeletal protein dynamics (actin; Wang, 1985, myosin; Mittal et al., 1987, McKenna et al., 1989, α -actinin; Feramisco, 1979, profilin; Cao et al. 1992, tubulin; Soltys and Borisy, 1985, intermediate filament proteins; Vikstrom et al., 1989) (for comprehensive review, see Taylor et al., 1986; Wang, 1989). This technique, however, had not been successfully applied to *Dictyostelium* amoebae until recently, due to their small size, unstable cell-substrate attachment, and stickiness to the needle.

The purpose of this chapter is to describe exact step-by-step protocols for incorporating conventional myosin (myosin II) into live *Dictyostelium*. As described below, we have a good luck in using Narishige Micromanipulator and Injectors for studying the myosin dynamics (Chu and Fukui, 1996).

II. Materials and Instrumentation

(A) Cells and medium

Dictyostelium discoideum, axenic strains are cultured in a modified HL5 medium at 22 °C.

To make 1 liter of the modified HL5 medium

Thiotone™ E peptone (Becton Dickinson; 12302) (Baxter; 12302 BT)	5 g
Proteose peptone, type 2 (Difco; 0121-17-5) (Baxter; 0121-01 GB)	5 g
Yeast extract (Oxoid; L21) (Unipath Company; LP021B)	5 g
D-[+]-Glucose (Sigma; G-5146)	10 g
Na ₂ HPO ₄ (Sigma; S-9763)	185 mg
KH ₂ PO ₄ (Sigma; P-5379)	350 mg

Adjust pH to 6.5 with 1 N HCl.

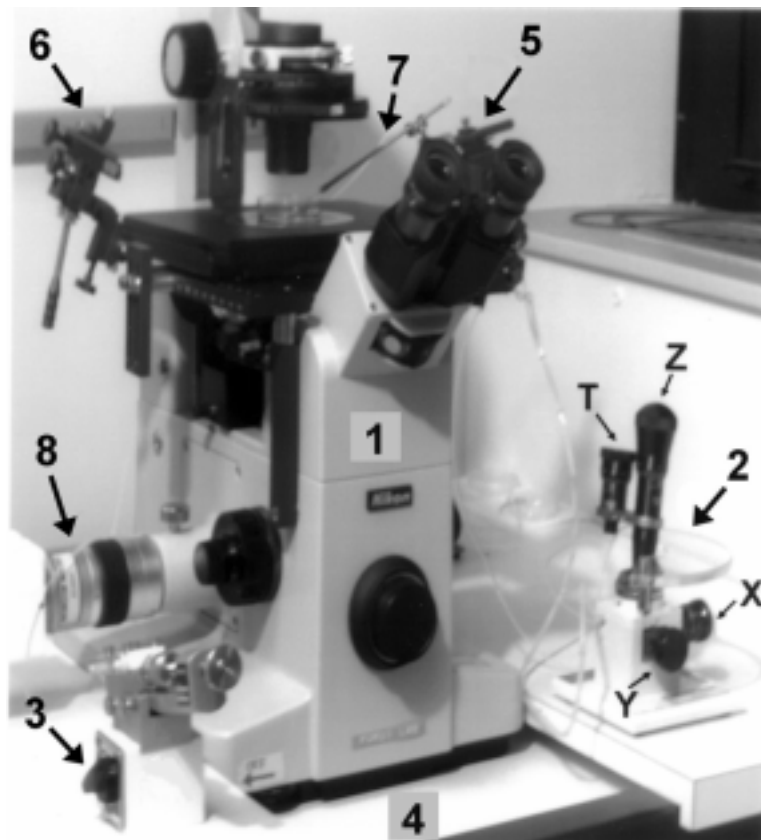
Autoclave for 20 minutes at 120 °C, and store in refrigerator.

(B) Rhodamine-myosin

Myosin is purified from vegetative stage amoebae (Mockrin and Spudich, 1976) and labeled with tetramethylrhodamine iodoacetamide (IATR) (McKenna et al., 1989). In average, we purify 20 mg of myosin from 300 g fresh weight of the amoebae cultured in a total of 90 liters of HL5 medium. We dissolve 2.5 mg of TR-myosin in 500 μ l injection buffer, made of 450 mM KCl in 10 mM PIPES (pH 7.0). **In fact, the recipes for good injection buffer are most critical. It is a balance between the nature of proteins (or other chemicals) AND the durability of the cells. What we know sure is that *Dictyostelium* does not like Na⁺ higher than 50 mM.**

(C) Microscope

We place an inverted microscope (Nikon; model TMD) on a “Vibraplane” Inertia Isolation Table (Kinetic Systems, Inc.; #1201) (**Fig. 1**). During the operation, the table is floated from the main frame by high-pressure nitrogen gas. The gas is supplied from a high-pressure tank, and the pressure is adjusted to 80 PSI with a regulator.



(Legend to Figure 1) System diagram. (1) Inverted microscope (Nikon; model TMD). (2) Joystick Hydraulic Micromanipulator (Narishige; model MO-202); (x, y) thimbles, (z, t) knobs. (3) Microinjector (Narishige; model IM-5B). (4) Floating table (Kinetic Systems, Inc.; “Vibraplane” Inertia Isolation Table, model 1201). (5) Remote-control Hydraulic Microdrive (Narishige; model MO-22). (6) Auxiliary light-weight manipulator

(Narishige; model M-4). (7) Microneedle holder (Narishige, model HI-4). (8) A zoom lens for the video camera .

(D) Micromanipulator

We use a Joystick Hydraulic Micromanipulator capable of four degrees of freedom (Narishige; system MO-204) (**Fig. 1**). This system consists of two units: (1) a base unit, model MO-202, and (2) a remote microdrive, model MO-22. We place the base unit on a sliding shelf fixed to the main frame of the floating table, while attaching the microdrive to the microscope stage with an adapter (Narishige; model NS-A).

(E) Microinjector

We use a Pressure Microinjector (Narishige; model IM-5B) in our laboratory (**Fig. 1**). The micropump unit is attached with a 3 ml glass syringe, which is connected, to the micropipette holder with ca. 50 cm long teflon tube (CT-1; O. D., 2 mm, I. D., 1 mm). The micropump is placed on a sliding shelf fixed to the other side of the microscope. One rotation of the pressure control knob brings in a displacement of 0.50 mm of the plunger, or 40 μ l of the content.

(F) Microneedles

The **Cored Glass Tubes** (Narishige; GD-1: I. D., 0.75 mm x O. D. 1 mm x length, 90 mm) are our favorite needles. The capillary is made of hard glass and contains a 100 μ m wide glass fiber, which helps smooth filling of the sample into the microneedle. We have also found that Capillary Tubing with Omega Dot Fiber (FHC: Frederick Haer & Co., Brunswick, ME; cat. no. 30-31-0, I. D., 0.9 mm x O. D. 1.2 mm) is usable; but Glass Thin Wall Capillary with Filaments (WPI: World Precision Instruments, Inc., Saratoga, FL; cat. no. 17324, I. D. 1.0 mm) are a bit thicker for our applications. After pulling the needle, we store them in a needle storage bin (WPI: Electrode Storage”, cat. no. E220).

We make microneedles using a **Glass Microelectrode Puller** (Narishige; model PN-3). We noticed that the microswitch contact on the left end of the rod needs to be regularly cleaned and lubricated otherwise the trigger becomes malfunctioned (see section III-B for detail).

(G) Locator Coverslips

One way to locate the microinjected cell is etching the coverslip using an **Object Marker** (Leitz, #513-442). This tool looks like an objective lens, and attached to one of the slots for objective lenses. A diamond tip is installed on the tip of this tool (instead of lens) with some misalignment such that, with light pressure on coverslip, rotation of the tool engraves a circle of 2-3 mm in diameter. We can also draw straight line by moving the coverslip.

We glue a locator coverslip placed in the center of a 60 mm x 15 mm plastic Petri dish (Becton Dickinson, Falcon #1007) using a very little amount of vaseline, put to a corner using a toothpick. Then we add 3.5 ml of the HL5 medium in the dish.

For microinjection, we use 20x or 40x objective lens, but for fluorescence observation, the sample will be observed using a Zeiss 63x (N.A. 1.4) or 100x (N.A. 1.3) plan apo objective lens. Therefore, we use no. 1-1/2 coverslips whose thickness is about 0.18 μ m.

Commercially available graded coverslips (Corning Glass, Co.) are made from no. 2 coverslips and found too thick to be used for these high-power objective lenses.

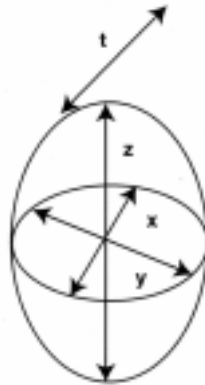
After injecting into 10-20 cells, we pick the coverslip from the dish (using forceps), rinse with salt solution, and mount on an observation chamber (Chu and Fukui, 1996). We can easily relocate injected cells based on the position relative to engraved marks. We can also identify the injected amoebae under a fluorescence microscope. For this purpose, we take advantage of the second dye to be used as a volume marker, for example FITC-dextran (see **section IV** for detail).

III. Setting Up

(A) Micromanipulator

The **Narishige Joystick Hydraulic Micromanipulator** is capable of linear, uni-directional movement by rotating a knob (t), in addition to the three-dimensional orthogonal, x, y, and z-axes, movement with a joystick and thimbles (**Fig. 2**). This feature allows a non-destructive, linear penetration of the microneedle. The ratio between the joystick movement and displacement of the microdrive is adjustable in the range of 600 :1 to 66 :1. The maximum distance of the movement of the thimbles and the knobs is 10 mm, and the minimal scale is 5 μm .

The position of the thimbles and the knobs should be set to zero when the system is not used. At this position, the hydraulic pressure is neutral and thereby a chance of possible oil leakage from the system is minimal. You must, however, re-adjust and re-tighten the force adjustment and the working distance adjustment rings frequently.



(Legend to Figure 2) A diagram showing the four-degree freedom provided by the Narishige MO-204 Micromanipulator system.

(B) Microneedle

Using the **Narishige GD-1 capillary**, our optimum settings for the Heater, Magnet and Main magnet adjustment dials are at positions 5, 4, and 3, respectively. Each time we pull more than ten microneedles and inspect them under a microscope using a 40x objective. We select smooth-tipped microneedles with a 0.3-0.5 μm tip diameter and store

them in a Microneedle Storage Container (WPI; # E-210). We also use a 8x magnifier (Edmund Scientific, # 35674) for a quick inspection of the needles. Using the **Glass Electrode Puller** (Narishige; model PN-3), shape of the microneedle can be adjusted by changing the position of the cut-off microswitch located at the end of the metal rod. We position the adjustment ring at 0.23 mm from the end of the rod.

A specific problem with our microelectrode puller is the occasional bad contact at the microswitch located at the end of the metal rod. When this happens, the heater will not be turned off even after it is completely pulled and the heating element keeps glowing. We have to turn the power switch of the unit off to prevent the platinum element from burning out. To solve this problem, we open the metal cover and lubricate the microswitch with electronic spray (Radio Shack; # 64-4315).

It is also important to inspect the shape of the heating element each time before you make microneedles. The element is made of thin fragile platinum and if it does not surround the capillary uniformly, a cross section of the pulled microneedle becomes asymmetric. To correct this problem, a filament-shaping tool (metal rod, diameter 2.5 mm) comes with the PN-3 unit.

(C) Microinjector

For precise pressure injection, it is critical to replace the air in the syringe and the entire tubing with inert oil. We use **Sigma's Mineral Oil** (M-5904) with great success. Care should be taken to remove air bubbles from the system. We “bleed” air bubbles by (1) filling a disposable 5 ml plastic syringe with the oil, (2) connecting the tubing to the syringe, and (3) loading the tube with oil by plunging the syringe slowly, with the free end of the tube lifted. Once the mineral oil is filled, we must firmly tighten hubs connecting the tubing and glass electrode (CI-1, CI-2, respectively) with a teflon gasket attached.

IV. Procedures

(A) Loading into a microneedle

We load the microneedle with 5 μ l of FITC-dextran (1 mg/ml; Sigma, # FD-4) using a long, flexible, non-metallic syringe needle (“MicroFilTM”; WPI, # MF34G). FITC-dextran serves as a locator of injected cells. The “MicroFilTM” is constructed from a combination of plastic and fused silica with a tip diameter of 0.164 mm and 70 mm long. Before loading the sample solution into the microneedle, we remove any precipitate from the sample solution by centrifugation (14,000 rpm, 5 minutes on an Eppendorf Centrifuge). We fill the microneedle about 0.5 cm from the tip using the “MicroFilTM”, and attach to a 130 mm long stainless micropipette electrode/injector holder (Narishige; model HL-4) fixed on the remote microdrive unit.

(B) Practicing Injection

Load the microneedle with 5 - 10 μ l of 5 mg/ml FITC dissolved in physiological buffer and fix the needle to the holder on the remote microdrive. Place a dish on the microscope stage and using a 20x objective, focus and find the position of locator marks. Locate several amoebae, and bring the tip of the microneedle close to an amoeba. To do this step efficiently, first use your fingers, next the joystick, and finally the thimbles and knobs. An

optimum angle of the needle to the coverslip is about 40 degrees in our hands. Change the objective to 40x, refocus on the amoeba, and fine tune the position of the tip. After placing the tip a few μm from an amoeba, simultaneously rotate the injector's pressure control knob and the t-direction knob clockwise. When the tip of the needle penetrates into the amoeba, pull it back. We can inject 10 - 20 amoebae around a locator marker within a period of 5 -10 minutes. Video showing the injection process is available on request.

Sometimes, cell debris attaches to the needle. To remove the debris, lift the needle to the surface of the medium and tap the microscope stage a few times. If the debris is still attached, using your fingers, flip the tip of the needle up and down several times, passing through the air-medium interface. The surface tension will rid the debris efficiently. Using a rubber bands, we also provide a continuous, low pressure to the injector to prevent possible clogging of the needle. Inject 10 - 20 amoebae around the locator marker, and lift the microneedle.

When the injection was successful, the amoebae should recover from the initial rounding up within 1-2 minutes, and start moving after about 3 minutes. The FITC-dextran must be diffusely distributed with its highest intensity at the perinuclear region.

(C) Example of Fluorescence Analogue Cytochemistry: Microinjection of TR-Myosin/FITC-Dextran

We load the microneedle with 5 μl of 1 : 1 mixture of TR-myosin (5 mg/ml) and FITC-dextran (1 mg/ml; Sigma, # FD-4). We monitor the dynamics of injected TR-myosin using a cooled CCD camera (Photometrics, model PXL) attached to a Zeiss Axioskop Fluorescence Microscope. We alternately acquire TR-myosin, FITC-dextran, and bright field images. The images are saved as 12-bit tiff files and analyzed with MetaMorph Image Acquisition and Analysis System (Universal Imaging Corp.) Results showing the myosin dynamics can be seen in our Home page at: <http://pubweb.nwu.edu/~yoshifk/fukui.html>.

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SUPPLIERS

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