Chapter 2

Single-Cell Microinjection Coupled to Confocal Microscopy to Characterize Nuclear Membrane Receptors in Freshly Isolated Cardiomyocytes

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Abstract

Lipid bilayers, such as the plasma membrane and nuclear envelope, serve as effective cellular barriers to ions and macromolecules, thus allowing regulated access to subcellular compartments including the cytoplasm and nucleus, respectively. Of course, these barriers are semipermeable and a wide variety of proteins including transporters, ion exchangers, pumps, and ion channels are required to permit access as well as establish and maintain molecular and ionic gradients across membranes. However, some experimental designs, such as specifically targeting intracellular receptors, require the administration of membrane-impermeable molecules directly into live cells. The microinjection technique described in this chapter is an efficient, technically simple, and reliable approach that can be used to introduce macromolecules into intracellular compartments while maintaining the integrity of the plasma membrane itself.

Key words Microinjection, Nuclear receptor, Confocal microscopy

1 Introduction

First reported in the early twentieth century [1], microinjection has been more commonly used in the last few decades to introduce macromolecules and cell-impermeable molecules directly into the cytoplasm or nucleus. For example, this approach has been used to inject genetically manipulated embryonic stem cells into enucleated blastocysts [2]. A wide variety of molecules, ranging from proteins to DNA constructs, can therefore be introduced in cells using this versatile technique. In addition to being exceedingly efficient, its reliability allows the experimenter to inject a defined quantity of material and then quantify experimental outputs.

Investigation of intracellular receptors such as the inositol 1,4,5-phosphate receptor (IP_3R) can be carried out by cytoplasmic application of IP_3 or photosensitive caged IP_3, which can release IP_3 with a high spatial and temporal resolution and subsequently

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activate IP_3R [3]. Similarly, identification and characterization of nuclear receptors in intact cells can be facilitated by microinjecting the ligand. Indeed, discrimination between internalized ligand-receptor complexes and the nuclear receptor can be achieved with microinjection of the ligand, as plasmalemmal receptors will not have been exposed to the ligand. For example, nuclear AngII has been investigated using this approach [4].

This chapter describes the microinjection of fluorescently labeled endothelin-3 (ET-3) into freshly isolated adult rat cardiomyocytes to allow the study of intracellular pools of the ET receptor.

2 Materials

Microinjection is performed using a Nikon Eclipse FN1 upright microscope as part of an Andor Revolution confocal system. Equivalent systems are available from other commercial sources. The use of two lasers (488 and 561 nm) and the appropriate emission filters permits concomitant injection of two different fluorophores. Images are acquired with an iXon EMCCD camera with a 60× water-dipping objective (N.A. 1.0) (*see* **Note 1**).

Air-pressure microinjection unit such as a FemtoJet system coupled to an InjectMan micromanipulator (Eppendorf) (*see* **Notes 2** and **3**).

Femtotip (Eppendorf) microinjection pipettes to access the cytosol and inject the molecule of interest (*see* **Note 4**). The microfilament in the pipette ensures that the solution reaches the tip of the microinjection pipette.

Microloader pipette tips to load the microinjection pipette with the desired solution.

All solution should be made at room temperature and prepared using fresh, type 1 deionized water (dH_2O).

Microinjection solution: 20 mM HEPES, 3 mM KCl, and 2 mM NaCl. The solution must be prepared fresh and, following pH adjustment (pH 7.2; with KOH), be filtered to 0.22 μm (*see* **Note 5**).

Extracellular solution (KB): 10 mM HEPES, 20 mM KCl, 10 mM KH_2PO_4, 70 mM K^+-glutamate, 100 mM K-aspartate, 2 mM $MgSO_4$, 5 mM creatine, 1 mM $MgCl_2$, 25 mM glucose, 10 mM β-hydroxybutyric acid, 20 mM taurine, 0.5 mM EGTA, and 0.1 % albumin. Adjust pH to 7.25 with KOH. KB solution can be stored at 4 °C for up to 7 days.

Laminin-coated cover slips: Prior to coating the cover slips, dilute laminin stock solution (1 mg/mL) to a concentration of 15 μg/mL using dH_2O. Apply and spread 100 μL of diluted laminin onto the cover slip. After incubating for 20 min at room temperature, remove the excess laminin solution. Cover slips must be completely dry before plating cardiomyocytes.

Rhodamine ET-3: Dilute rhodamine ET-3 in the microinjection solution to achieve a final concentration of 2 nM. Keep on ice.

Sodium fluorescein solution: Dilute sodium fluorescein to a concentration of 0.2 mM in microinjection solution.

Syto 11: Fluorescent nucleic acid stain that is used to label the nucleus.

3 Methods

3.1 Cardiomyocyte Isolation

Freshly isolated adult rat ventricular cardiomyocytes were prepared as described previously [5, 6]. Following enzymatic dissociation of ventricular cardiomyocytes, centrifuge cells for 1 min at 45 × *g* at room temperature. Remove isolation buffer with gentle aspiration and replace with 10 mL KB buffer. Repeat this wash step three times to remove all traces of collagenase from the cell suspension. This step increases longevity and viability of the harvested cells (*see* **Note 6**). Resuspend cardiomyocytes in KB, plate 300 μL of cells on a laminin-coated cover slip, and incubate for 1 h at 37 °C in a humidified chamber. It is important to aim for a final confluence of ≈50 %. Cells must be used within 6 h of isolation.

3.2 Microinjection

When filling the Femtotip microinjection pipette, select the "capillary exchange" mode in the menu of the FemtoJet system, and then connect the injection tube. Using a Microloader, insert 1–5 μL of microinjection solution into the microinjection pipette (*see* **Note 7**). Then, place the microinjection pipette on the pipette holder on the InjectMan, secure the pipette, and press Menu to exit from "capillary exchange" mode on the FemtoJet.

Using rhodamine-conjugated ET-3 allows the use of ET-3 as a fluorescent marker for intracellular ET_B receptors. Combined injection of fluorescein and rhodamine-ET-3 enables the operator to assess the efficiency of microinjection (*see* **Notes 8** and **9**).

Prewarm the solutions and the experimental chamber. Experiments are performed at 37 °C.

Using forceps, carefully transfer a cardiomyocyte-loaded cover slip in the recording chamber (*see* **Note 10**).

Visually identify a healthy, quiescent cardiomyocyte (*see* **Note 11**) in the chamber using bright-field illumination (Fig. 1). Position the microinjection pipette beside (not above) the cell. Adjust the focal plane of the microscope to be at approximately half of the height (*z*-axis) of the cell. Now, move the microinjection pipette until the tip is in focus at this focal plane. The microinjection pipette is now aligned with the midpoint of the cell (on the *z*-axis). Set this *z* value as the "z limit" on the InjectMan with the option "inject at z limit." This will be the height at which the microinjection will be initiated by the system. Setting the z limit too high will result in a missed injection (release of the solution above the cell).

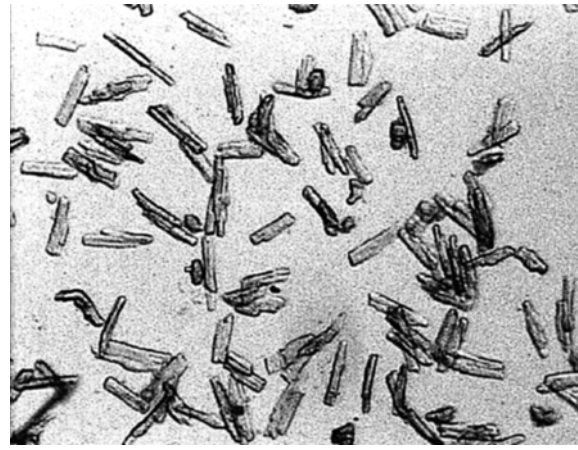

Fig. 1 Freshly isolated rat ventricular cardiomyocytes. Typical field of view of freshly isolated ventricular cardiomyocytes seeded on a laminin-coated cover slip and viewed using bright-field illumination. Note the distinctive *rectangular* shape of the cells

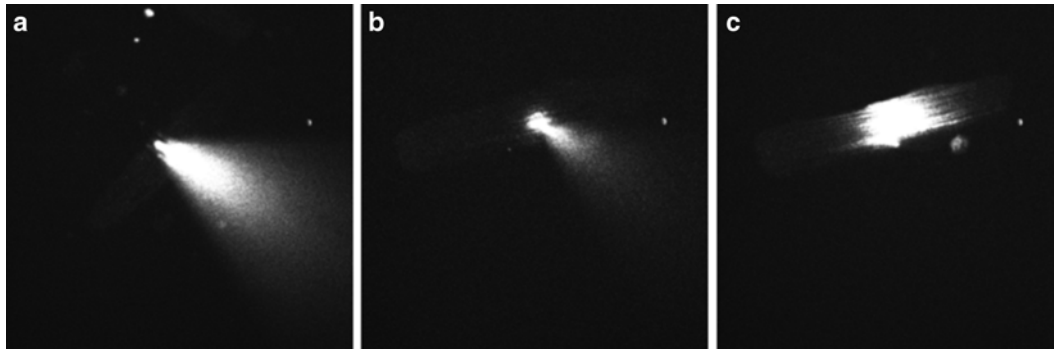

Fig. 2 Microinjection of rat ventricular cardiomyocytes with fluorescein and rhodamine-ET-3. Representative images selected from a typical microinjection experiment of a rat ventricular cardiomyocyte. Confocal images of a cardiomyocyte taken prior to (**a**), during (**b**), and 45 s following (**c**) microinjection

In contrast, using a z limit that is too low might result in the micropipette coming in contact with the bottom of the chamber and breaking (*see* **Note 12**).

Raise the focal plane of the microscope until the top of the cardiomyocyte is in focus. Now, raise the microinjection pipette until the tip is slightly above the focal plane. Finally, maneuver the microinjection pipette until the tip is above the cell at the exact *x*–*y* value where the microinjection will be done (*see* **Note 13**). At this point, begin recording confocal images. Pre-injection images will serve as "control" or "basal" values. Finally, initiate the microinjection by pressing on the joystick button (InjectMan) while continuously recording images to track injected fluorophores within the cell (Fig. 2).

The previous steps can be repeated on the same slide as long as healthy cardiomyocytes are available in the chamber given the microinjection pipette is not clogged.

For each cell, at the end of the experiment, add Syto 11 to the chamber at a final concentration of 1 μM to label the nucleus. This can be used to compare, for example, ET-3 staining to Syto 11 and show the localization of endothelin receptors relative to the nucleus (*see* **Note 14**).

4 Notes

1. Although we use an upright microscope, microinjection can also be carried out using an inverted microscope. In such cases, microinjection is easier since the objective lens of the microscope does not interfere with the microinjection system. Moreover, on an inverted microscope, the microinjection manipulator is usually attached to the body of the microscope and will stay in position with the objective while the chamber is moved around to find the best cells to inject. Alternatively, the platform where the micromanipulator of an upright scope is sitting is the same as that holding the chamber. Therefore, the microinjection pipette will move with the chamber when searching for a cell and may move out of view when the chamber is repositioned. However, an upright microscope is very useful when working with multicellular preparations and tissues, such as brain slices and whole arteries, where the cells to be imaged are located on the surface of the tissue.
2. The use of two micromanipulators, one to microinject and the other one to hold the cell, is not required in the described protocol. The use of laminin (Cell-Tak is a potential alternative) on the cover slip allows cardiomyocytes to attach to the cover slip and remain immobilized during microinjection and image acquisition. However, the use of laminin-coated plates does not prevent spontaneous contraction of cardiomyocytes.
3. The angle of the microinjection pipette is to be set between 30° and 45°. Although an angle of 45° is often preferred, with an upright microscope a different angle might be required due to the objective lens impeding access of the micropipette approaching the targeted cell.
4. In order to avoid the high cost of buying microinjection pipettes, custom-made pipettes can be generated from borosilicate glass capillaries using a regular pipette puller. However, significant optimization is required to find the best settings for pipettes to be used for microinjection. Depending on the pipette puller used, several parameters

might need to be optimized, including heating temperature, time, pulling force, and sequence of the protocol.

5. Filtration of the microinjection solution is very important to ensure that the microinjection pipette does not become clogged with debris during experiments.
6. It is important to carefully remove any phenol from the isolation buffer, as phenol interferes with fluorescence and will alter the fluorescence recordings.
7. When filling the microinjection pipette, bring the Microloader tip close to the tip of the microinjection pipette and carefully release the contents of the Microloader. Then gently flick the microinjection pipette with your finger to bring any air bubbles to the top of the microinjection solution. Air bubbles can impede injection and must be removed from the microinjection pipette. However, care must be taken here as the microinjection tips are extremely fragile.
8. Briefly centrifuge the rhodamine-ET-3 stock solution in a microcentrifuge just before diluting it in microinjection solution to avoid blocking the microinjection pipette. Co-injecting rhodamine-ET-3 with fluorescein allows the operator to assess (1) loading of the microinjection pipette, (2) the quality of the microinjection (e.g., fluorescein will reveal the entry and diffusion of the microinjection buffer through the cell as well as any leakage at the site of injection), (3) the compensation pressure (*see* below), and (4) if the pipette is blocked.
9. In the current protocol, fluorescein was chosen to be co-injected with rhodamine-ET-3 as it permits concomitant recording. Alternatively, if the ligand of interest is conjugated with a dye that requires excitation in the 488 nm range, a dye such as Texas Red can be co-injected, depending on the laser/filter availability of the imaging system. However, autofluorescence can be a problem when cardiomyocytes are excited by a 488 nm laser, especially when using low-quantum-yield dyes or studying low-density target proteins.
10. Compensation pressure is an important parameter that must be set prior to performing actual experiments. The compensation pressure is defined as the pressure applied by the system to compensate for the differential force of the capillary suction and hydrostatic pressure. The compensation pressure can be determined with a fluorescein-loaded microinjection pipette. Using the confocal system, while looking at the tip of the microinjection pipette, adjust the compensation pressure on the FemtoJet system to ensure that there is no leak of fluorescent solution. Then, injection pressure and time should be set, again under control conditions with only fluorescein in the

pipette. Once the different pressure and duration parameters defined, the injection volume can be determined. Load a microinjection pipette with 1 μL of solution and repeatedly activate the injection until the microinjection pipette is empty. The volume of each injection can then be assessed by dividing 1 μL by the number of injections required to empty the pipette.

11. Quiescent (non-beating) cardiomyocytes are considered healthy cells as their Ca^{2+} homeostasis mechanisms remain able to prevent Ca^{2+} overload and cell death. Unhealthy cardiomyocytes develop blebs on their surface and then hypercontract. Moreover, moving cardiomyocytes are extremely difficult to microinject without either damaging the microinjection pipette or the target cell.
12. To a neophyte, an unexpectedly delicate part in the microinjection procedure is to locate the tip of the microinjection pipette in order not to crush the tip against the bottom of the chamber. Use a lower magnification objective lens to find the pipette when it first enters the solution. The halo created by surface tension can be used to quickly localize the tip of the microinjection pipette. Once found, the tip can be lowered into the chamber while simultaneously adjusting the focus to be ahead of the tip. When the outline of the cells becomes visible, stop lowering the microinjection pipette and change the step size of the micromanipulator to the fine or extra-fine setting.
13. In experiments where the objective is to study nuclear receptors, it is important not to aim the microinjection pipette at the nucleus of the cell (during injection, the microinjection pipette may actually damage the nucleus). Hence, when positioning the microinjection pipette in the *x*–*y* plane over the cell, choose an injection site that is not too close to the nuclei (adult cardiomyocytes are usually binucleate). For example, the microinjection site can be halfway between the two nuclei. The nuclei are clearly visible when using bright-field illumination.
14. Although the nuclei in cardiomyocytes can be identified as elliptical regions devoid of fluorescence from the other injected fluorophores, the use of a cell-permeable nucleic acid stain such as Syto 11 is a preferable means of identifying nuclei. Syto 11 has an excitation/emission of 508/527 nm and can therefore be viewed with a 488 laser and filters as for FITC.

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