Video Article

Activation of Apoptosis by Cytoplasmic Microinjection of Cytochrome *c*

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Abstract

Apoptosis, or programmed cell death, is a conserved and highly regulated pathway by which cells die[1]. Apoptosis can be triggered when cells encounter a wide range of cytotoxic stresses. These insults initiate signaling cascades that ultimately cause the release of cytochrome *c* from the mitochondrial intermembrane space to the cytoplasm[2]. The release of cytochrome *c* from mitochondria is a key event that triggers the rapid activation of caspases, the key cellular proteases which ultimately execute cell death[3-4].

The pathway of apoptosis is regulated at points upstream and downstream of cytochrome *c* release from mitochondria[5]. In order to study the post-mitochondrial regulation of caspase activation, many investigators have turned to direct cytoplasmic microinjection of holocytochrome *c* (heme-attached) protein into cells[6-9]. Cytochrome *c* is normally localized to the mitochondria where attachment of a heme group is necessary to enable it to activate apoptosis[10-11]. Therefore, to directly activate caspases, it is necessary to inject the holocytochrome *c* protein instead of its cDNA, because while the expression of cytochrome *c* from cDNA constructs will result in mitochondrial targeting and heme attachment, it will be sequestered from cytosolic caspases. Thus, the direct cytosolic microinjection of purified heme-attached cytochrome *c* protein is a useful tool to mimic mitochondrial cytochrome *c* release and apoptosis without the use of toxic insults which cause cellular and mitochondrial damage.

In this article, we describe a method for the microinjection of cytochrome *c* protein into cells, using mouse embryonic fibroblasts (MEFs) and primary sympathetic neurons as examples. While this protocol focuses on the injection of cytochrome *c* for investigations of apoptosis, the techniques shown here can also be easily adapted for microinjection of other proteins of interest.

Video Link

The video component of this article can be found at http://www.jove.com/video/2773/

Protocol

1. Production of Microinjection Needles

1. Pre-fabricated microinjection needles are available commercially (*e.g.* Femtotips from Eppendorf) and are useful if one is not performing a large number of microinjections. However, for those who wish to establish long-term capabilities for microinjecting, an alternative is to produce microinjection needles in the lab using thin wall borosilicate glass capillaries and a commercial needle puller. This also allows the shape of needles to be varied, which can be useful for different cell types.
2. With the Narishige PC-10 Microinjection Needle Puller, attach all four weights and use a one-step pulling program (Step 1 setting) with the relative heat setting at 58.0 (No.2 heater). Be sure to place the heating element at the center of each capillary so that the two resulting needles are a similar length.
3. Pull several capillaries (about 2 capillaries for each protein of interest).
4. Store needles in a container while being sure not to damage the needle tip. Materials such as foam or Blu-Tack can be used in containers to hold microinjection needles.

2. Preparation of Protein Mixtures for Injection

1. Prepare a 10x microinjection buffer containing 1 M potassium chloride and 0.1 M potassium phosphate (KPi) buffer (equimolar mixture of K_2HPO_4 and KH_2PO_4) at a pH of 7.4. This buffer can be stored long-term at room temperature.
2. To visualize the injection, a fluorescent dye like rhodamine-dextran needs to be added. Dilute the10x microinjection buffer in water and dissolve rhodamine-dextran powder to make a 5x microinjection buffer solution containing 20-40 mg/mL rhodamine dextran.
3. Store this solution in the dark at 4°C. It should be sufficient for up to 100 individual protein solution preparations. Other dyes, like fluorescein isothiocyanate-dextran can substitute.

4. Prepare cytochrome *c* stocks by dissolving purified cytochrome *c* in water to a concentration of 20 mg/mL. Store cytochrome *c* at -80°C for long-term storage and avoid freeze-thaw cycles by storing cytochrome *c* in small (~10 µL) aliquots.
5. Prepare a 10 µL protein mixture for injection by combining 2 µL 5x microinjection buffer containing rhodamine-dextran with 3 µL water and 5 µL cytochrome *c* for a final concentration of 10 mg/mL cytochrome *c* in 1x buffer (100 mM KCl, 10 mM KP_i, 4-8 mg/mL rhodamine dextran).

3. Cytoplasmic Microinjection of Cytochrome *c*

1. Just prior to microinjection, centrifuge the mixture of cytochrome *c* and microinjection buffer at 16,000 g for 10 minutes at 4°C to separate any particulate matter which may clog microinjection needles.
2. During centrifugation, turn on the microinjector to allow air pressure to build.
3. Place a dish of cells on the center of the microscope stage and set the focus on the cells. To minimize the amount of time that the cells are kept outside the incubator, cells are typically returned to the incubator within 30 min.
4. Pipette 0.5-1 µL from the top surface of the protein mixture into the blunt end of the capillary. Be careful to not pipette any particles which have been centrifuged to the bottom of the tube. Within a minute, the protein mixture will distribute to the needle tip through capillary action.
5. Attach the needle firmly to the capillary holder of the micromanipulator and position the needle so that its tip passes through the transmitted light of the microscope at approximately a 45° angle.
6. Adjust the position of the needle tip so that it is located directly in the center of the field of view. To center the needle, use the micromanipulator to move the needle while looking through the microscope eyepiece. The needle shadow should be visible. Adjust the needle so that its shadow is only seen in one half of the field of view, indicating that the needle tip is centered above the cells.
7. Set the microinjector to the Continuous Flow mode and set the working pressure to 20 - 100 hPa. Each needle may require a different working pressure, and the working pressure will likely need adjustment during the microinjection procedure to maintain a steady flow.
8. Lower the needle using the coarse knob to a position just above the cells. To do this, raise the focal plane of the microscope to a position just above the cells. Then lower the needle towards the cells using the coarse knob until the needle tip is in focus.
9. Re-center the needle tip within the field of view and increase magnification by changing the microscope objective.
10. Slowly lower the needle using the fine manipulator knob until the needle is just slightly above the focal plane of the cells.
11. Check the flow of the protein mixture by looking at the red fluorescence of the rhodamine. The protein mixture should be exiting the needle as a thin, constant stream. Needles should be replaced and re-loaded if the needle is compromised or if the flow is far too strong. Sometimes, the tip of the glass capillary is closed and no flow is seen from the needle. If this occurs, replace the needle or carefully lower it to the bottom of the culture dish to gently rupture the tip of the needle.
12. Position the needle tip so that it is pointing towards a cell at approximately a 45° angle. Then with one smooth motion, lower the needle while moving it towards the cell. With a second smooth motion, immediately reverse the direction of the needle to remove it from the cell.
13. A successfully injected cell will often slightly swell and can be confirmed by visualizing the red fluorescent rhodamine within the cell. Occasionally, a cell will be accidentally injected in the nucleus, which will be visible.
14. Continue to inject cells by adjusting the microscope stage until about 50-100 cells are injected. When moving the microscope stage, be sure to raise the microinjection needle so that it clears the top of cells.

4. Representative Results:

The cytoplasmic microinjection of cytochrome *c* mimics its release from mitochondria during apoptosis. Thus, as expected, fibroblasts rapidly undergo apoptosis upon cytosolic microinjection of bovine cytochrome *c* (Fig. 1A). To ensure that the injection procedure alone is not responsible for cell death, injection of yeast cytochrome *c* serves as an important control, since yeast cytochrome *c* is incapable of activating caspases[12].

Interestingly, post-mitotic sympathetic neurons are remarkably resistant to cytosolic cytochrome *c* (Fig. 1B)[8,13]. Our lab has identified that the endogenous caspase inhibitor XIAP is a key inhibitor of caspase activation in neurons[14]. Thus, for neurons to die following cytochrome *c* injection, XIAP must first become inactivated. For example, microinjection of cytochrome *c* into $xiap^{-/-}$ sympathetic neurons is sufficient to allow caspase activation and apoptosis in these cells (Fig. 2).

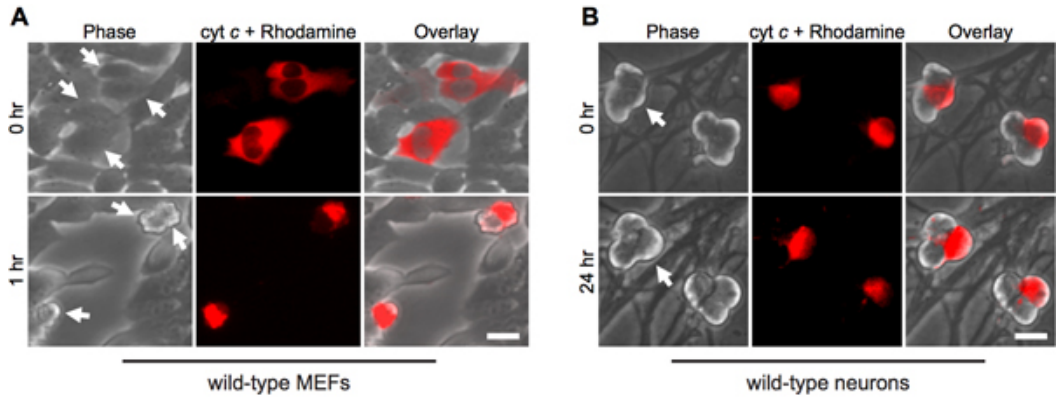

Figure 1. Cytoplasmic microinjection of cytochrome *c* induces rapid death in fibroblasts, but not neurons. A) Wild-type MEFs or **(B)** postnatal day 5 wild-type sympathetic neurons were microinjected with bovine cytochrome *c* (10 mg/mL) together with rhodamine-dextran to mark injected cells. Images show the same field of cells immediately following injection (0 hr), or at the indicated times. Arrows indicate injected cells. Scale bar, 20 µm.

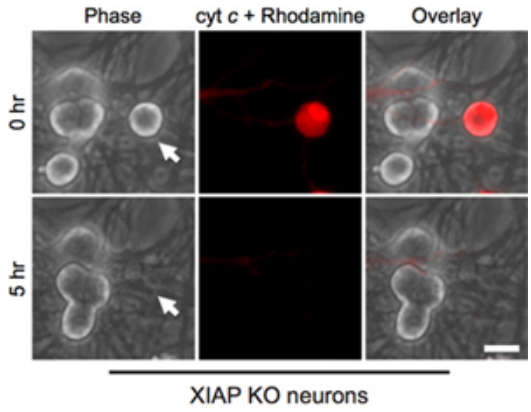

Figure 2. XIAP-deficient neurons are susceptible to cytoplasmic cytochrome *c* microinjection. Postnatal day 5 sympathetic neurons from XIAP knockout mice were microinjected with bovine cytochrome *c* (10 mg/mL) together with rhodamine-dextran to mark injected cells. Images show the same field of cells immediately following injection (0 hr), or 5 hours after cytochrome *c* microinjection (5 hr). Scale bar, 20 μm.

Discussion

The microinjection of cytochrome *c* directly into the cytoplasm of cells is a unique and powerful tool which allows for studies of the post-mitochondrial regulation of apoptosis. Importantly, this technique allows for the direct activation of apoptosis downstream of mitochondria without the use of agents which cause cellular or mitochondrial damage.

While this protocol has focused on microinjection of cytochrome *c* for studies on apoptosis, the general principles of protein microinjection shown here can also be used for other proteins of interest. For example, some investigators have used microinjection of antibodies that target specific proteins, such as cytochrome *c* or c-Jun in studies of apoptosis[13,15].

The most common difficulty during microinjection is the clogging of microinjection needles during the procedure. If the needle becomes clogged, one can use the “clean” function on the microinjector which sends a strong pressure pulse through the needle to expel particles blocking the needle opening. Oftentimes, cleaning the needle is sufficient to unclog the needle. However, if dye is seen exiting the needle during a “clean” but immediately stops again, this indicates that the working pressure on the microinjector may be too low. In this case, increase the working pressure until a continuous flow from the needle is seen again. Cleaning the needle does not always restore flow, in which case the needle most likely needs to be replaced. One technique which can be used as an alternative, especially if the injection material itself is precious, involves gently breaking the tip of the microinjection needle against the bottom of the tissue culture dish. If done carefully, this can enlarge the opening of the needle and will restore flow. However, a large crack in the needle opening will cause a massive release of dye into the culture dish, obscuring the view of cells.

Unfortunately, not all cell types are capable of being microinjected. Some cells (e.g. cerebellar granule neurons) are too small for microinjection. Other cells (e.g. adult cardiomyocytes), while large enough, cannot withstand the injection procedure and die even with control injections. As described above, the microinjection of yeast cytochrome *c* serves as a useful control since yeast cytochrome *c* is unable to activate caspases. Cells microinjected with yeast cytochrome *c* will not activate apoptosis unless it is due to the microinjection procedure itself. Finally, some cells (e.g. fibroblasts) can withstand microinjection but can be difficult to inject because of their flat morphology. As a result, there is a risk that the microinjection needle will break against the bottom of the tissue culture dish with each cell that is injected.

Automated or manual micromanipulators can be used for protein microinjection, with each system having advantages and disadvantages. Automated micromanipulators are convenient because one can set a z-axis level to which the microinjection needle is automatically lowered for injecting cells. This is particularly useful when injecting cells whose height is similar across a cell culture monolayer. However, for cells which are cultured on coated dishes, (e.g. neurons plated on collagen-coated dishes), the three-dimensional nature of these cultures makes setting a specific z-axis level tedious. For these cultures, manual micromanipulators are advantageous since the needle can be rapidly adjusted to the specific height of each cell.

Microinjection can be a difficult technique to master and will require practice. In addition, the technique of microinjection has some limitations. For example, only a small proportion of cells in a tissue culture dish can be injected. Thus, biochemical preparations in which the whole culture is collected (e.g. cell lysates for Western blot) are not an accurate representation of the injected cells alone. Instead, most experiments need to be completed at the single-cell level. However, once the basic techniques presented here are mastered, one can perform a unique set of experiments to test hypotheses that cannot be answered using other methods.

Disclosures

All experimental procedures on animals were approved by the Institutional Animal Care and Use Committee at the University of North Carolina.

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