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Microinjection

Methods and Applications

Edited by

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Cover illustration: The image shows how a holding pipet is used to immobilize a zebrafish oocyte for microinjection. A typical holding pipet produced by flame polishing of a glass capillary is used to pick up and immobilize an unfertilized oocyte. Magnification is indicated by the bar which represents 100µm. Photo credit: William H. Kinsey.

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Preface

David J. Carroll

Abstract

Cellular microinjection techniques have developed over the last century along with the evolution of other biological fields. For example, developmental biologists have used a variety of microinjection techniques to transfer cytoplasm between cells, inject antibodies and peptides, and express foreign genes in specific tissues to advance their understanding of cell specification and determination. Molecular biologists finely craft glass microneedles and tools for the manipulation of cells in order to study gene expression and communication between cells. As these techniques have matured, microinjection has become accessible and, thus, exploited by a larger segment of the scientific and medical community. As more and more information is gathered from the various genome projects, demand grows for methods to validate these new data. Microinjection can help address this need. In particular, microinjection has proven valuable for the confirmation and extension of *in vitro* results in an *in vivo* setting – the living cell. The technique has also found a home in the clinical setting, most obviously within the community of fertility specialists for *in vitro* fertilization methods and for those excited about the possibility of therapeutic cloning.

This book explores the use of microinjection for a wide range of scientific uses. There are special considerations for each application of microinjection – whether it is the injection of antibodies, fusion proteins, DNA, and *in vitro*-synthesized RNA or the production of transgenic animals. It is hoped that these methods will be of interest for all biologists for use in the research laboratory, as well as for clinicians interested in applying this powerful method for treatment in the clinic.

1. A Brief History of Microinjection

The technique of microinjection was born of necessity and owes its history to a combination of fields. Credit for the initial description of a coherent microinjection technique could be given to Marshall Barber, who developed methods for producing fine glass capillary pipettes for isolating and manipulating single bacterial cells (1,2), or to the embryologist Laurent Chabry, credited with developing the glass microcapillary and micromanipulator as tools for his studies on teratology in ascidian blastomeres (3,4). Barber incorporated techniques into his injection method that are still used today, including the first use of mercury for controlling the movement of small volumes of fluid (see below) and the use of a second pipette to hold the cells as the injection is completed (5,6).

The technique of microinjection moved from being a useful method practiced by a few resourceful scientists to an exciting mainstream application when Gurdon and colleagues demonstrated that

purified mRNA from one cell could be injected into the cytoplasm of another cell and actually get translated into protein (7). This was remarkable for several reasons: (1) the mRNA was stable, at least stable enough that it was capable of directing the synthesis of detectable levels of protein; (2) no special factors, other than the exogenous mRNA itself, were required for this to work; and (3) success was independent of the cell type of the donor RNA. In fact, species differences do not appear to matter, as they were able to obtain the synthesis of rabbit hemoglobin in *Xenopus laevis* oocytes.

Since then, the method of expressing protein from microinjected mRNA has been used to great advantage in studies for developmental biology, neurobiology, cell biology, and signal transduction, just to name a few. An excellent overview of this method is given by Douglas Melton (8), in which he explains why the oocyte (and the *Xenopus* oocyte, in particular) provides the perfect system for this to work, along with describing details of the current methods at that time. One great advantage of this system, compared to translation in vitro or by induced expression in bacteria, is that the mRNA is placed in a living eukaryotic cell so the protein is processed properly and subject to post-translational modification.

Of course, the technique is not only useful for microinjecting RNA into *Xenopus* oocytes. It has been utilized for transferring cytoplasm from one cell to another, such as was done in the experiments that led to the discovery that maturation promoting factor (MPF) was neither species nor cell-type specific (9–11). This fundamental work provided the groundwork for the eventual discovery of cyclins and cyclin-dependent kinases (12,13). Proteins have been injected into cells for the study of cell structure and function. One of the first examples of this utility was the microinjection of fluorescently labeled α -actinin into living fibroblasts, allowing the visualization of this molecule integrating dynamically into the cytoskeleton (14). Of course, the concept of following labeled proteins in a living cell has since exploded with the availability of green fluorescent protein (GFP) vectors and protocols (15,16).

One tremendous advantage of microinjection, when compared to other methods of introducing material into cells, such as electroporation or chemical membrane permeabilization, is the ability to be quantitative. Methods for quantitative microinjection have developed over the years, but began in earnest with a study by Hiramoto on the process of fertilization (17). In that study, live spermatozoa were microinjected into sea urchin eggs to develop an assay for the identification of a “substance or substances which trigger the train of fertilization reactions in the egg”. No such substance was identified in that study (see **Chapter 2**), but methods to precisely control and quantify the volume of the injection using a mercury-filled needle connected to a screw-controlled

syringe were described. Hiramoto's basic method has been further refined (18–20), but is still being used today.

This book provides methods of microinjection coupled with modern molecular techniques, such as RNAi, morpholino antisense oligonucleotides, GFP expression, or the production of transgenic cells or animals, for example. However, the book also revisits classic uses of microinjection, such as mRNA expression or nuclear transfer, with modern twists.

2. Book Content

The classic technique of microinjecting *Xenopus* zygotes with mRNA prepared in vitro is revisited in **Chapter 1** with a focus on studying proteins involved in the cell cycle. A discussion of the use of microinjection compared to other methods of manipulating proteins in a living cell is given. The chapter also provides consideration of choosing the appropriate plasmid vector with an eye toward downstream analysis of the effectiveness of the protein expression. This discussion should be very useful for investigators considering the use of mRNA microinjection for the first time.

In **Chapter 2**, a very clever method using a luciferase chimera to visualize the expression of a protein, PLC ζ , that causes calcium release and egg activation during fertilization in mammals is described (21). Methods are given for producing the luciferase-labeled cRNA, along with techniques for assessing the expression of the fusion protein by microscopy, which allows simultaneous imaging of fluorescent indicators (e.g., a Ca²⁺ indicator), or by luminometer for quantification of luciferase expression. This method could easily be applied to other molecules in any cell type that will express exogenous RNA. In particular, it promises to be useful for combining detection of low levels of protein expression with quantification of those molecules from a reasonable number of cells.

In **Chapter 3**, a very straightforward technique for using antisense morpholino oligos to specifically remove 14-3-3 proteins in *Xenopus laevis* is described. Morpholino oligos are non-ionic DNA analogs that possess an altered phosphodiester backbone. This apparently makes them more resistant to nucleases and, because they are not charged, less likely to interact nonspecifically with proteins (22). This technique complements their earlier work in which the 14-3-3 proteins were studied using peptide inhibitors or dominant-interfering GST fusion proteins (23). Their method utilizes morpholinos that target the initiation codon and the 22 ribonucleotides immediately downstream,

which may produce more effective inhibition. This chapter outlines methods for (1) procuring the *Xenopus* sperm and eggs, (2) setting up and using a pressure microinjection apparatus with the *Xenopus* one- or two-cell embryo, (3) for analyzing and quantifying protein levels by western blotting, and (4) for analyzing the phenotypic effects of the morpholino injections. An alternate strategy for morpholino design that targets the 5' untranslated region of the target gene is also discussed.

Methods for microinjecting peptides and fusion proteins into *Xenopus laevis* oocytes are given in **Chapter 4**. Following a very clear description of ovary dissection and oocyte procurement by collagenase treatment, the procedure for performing rapid microinjections into the *Xenopus* oocyte is given. Accompanied by excellent photographs, this chapter explains the process in detail from start to finish. For example, in the text and in the Notes section, suggestions are given regarding the exact size for the tip of the microinjection needle and when the needle should be changed, the maximum volume and concentration of protein that should be injected into an individual oocyte, and how to deal with multiple injections into the same oocyte. This type of detail fills the chapter and will help other investigators maximize success when they adopt this method.

A method to combine microinjection with western blot analysis is described in **Chapter 5**. In this procedure, single oocytes are microinjected with a pharmacological inhibitor of RAS and then assayed for the presence of phosphorylated mitogen-activated protein kinase (pMAPK), which indicates an active enzyme, by immunoblotting. Thus far, this has been applied only for the analysis of the pMAPK during oocyte maturation and fertilization in starfish oocytes. However, it should also be useful in other large cells (such as *Xenopus* oocytes) and, as detection methods improve, it could easily be adapted to the analysis of other proteins in other systems. Analysis of single cells should prove useful because it eliminates the variability inherent to the analysis of cell populations.

This book considers the use of many different model systems, including the zebrafish *Danio rerio*. The zebrafish has proven extremely useful for experimental study because it is amenable to genetic studies, the embryo develops rapidly, and it is optically clear (you can see inside). The zebrafish zygote and early embryo have been utilized as a system for microinjection because of the large size of the eggs and the ease with which the adults are maintained. However, for a variety of reasons it would be advantageous to inject the mature egg prior to fertilization and this has proven difficult. In **Chapter 6**, a practical method for microinjection, and subsequent insemination, of the unfertilized zebrafish egg is reported. This will open up more opportunities for exploiting an already useful model system.

In the first method (**Chapter 7**) to deal with a ‘non-gamete’ system, techniques for antibody microinjection and oligofectamine transfection of RNAi for analysis of protein function in living tissue culture cells are compared. The chapter is very detailed which should allow these methods to be adapted to many different situations. The chapter also features a very extensive and useful notes section, detailing specifics of each of the methods.

Methods for developing recombinant cells lines by microinjection into the nucleus are presented in **Chapter 8**. This very interesting article demonstrates that introduction of plasmid DNA for the GFP, in several cell types, leads to stable transduction as assayed by flow cytometry of GFP fluorescence up to 1 month after microinjection! The chapter details methods that could be adapted to virtually any cell type and for any DNA. Conditions for optimizing the production of the transformed cells lines are tested in this chapter and detailed notes are given to help any investigator interested in attempting this method.

Different experimental methods, including transposons, *I-SceI* meganuclease, and direct injection of linearized DNA, have been used to produce transgenic *Xenopus* for the study of a variety of problems (24–26). However, these methods rely upon random insertion into the *Xenopus* genome or produce multiple copies. In **Chapter 9**, a technique is described for the targeted insertion of a single copy of the gene of interest into *Xenopus laevis* using phiC31 integrase. By incorporating insulator sequences into the plasmid design, they improve expression from the reporter gene making this method extremely useful for anyone wishing to express a transgene at approximately endogenous levels.

Transgenics are also the topic of **Chapter 10**. A method for producing transgenic *Caenorhabditis elegans* by microinjecting DNA directly into the hermaphrodite gonad is provided. For those not familiar with *C. elegans*, this chapter provides an excellent introduction to this powerful system. It recounts the basic reproductive biology of the worm and includes a discussion of applications for microinjection. Techniques for cultivating the worms and special hints for maximizing success of microinjection are explained in detail.

One of the great advantages of using microinjection to introduce molecules into living cells is the ability to perform quantitative experiments. **Chapter 11** gives detailed methods for the quantitative microinjection of picoliter quantities into mouse oocytes and eggs in dishes on an inverted microscope. The technique is described in wonderful detail and covers all aspects from making the glass bottom dishes that hold the oocytes to the injection process itself. There are several items of note in the chapter. I particularly enjoyed learning how to use an old record player to construct a beveler capable of producing a nice 1–2 μm tip. But then, what will I do with my old Pink Floyd records?

For precision, the microinjection needles need to include a mechanism for controlling the rate of injection. Two methods are given in **Chapter 11** to achieve this: (1) using mercury to backfill the micropipette and relying upon the mercury to transduce and control pressure and (2) constructing a microneedle with a constriction near the tip which reduces fluid movement and provides fine control of the injection. Both types of microinjection needles can be used in the same overall system.

A similar microinjection system is described in **Chapter 12**, with modifications that allow for injection of the mouse oocyte within the complete follicle. Because the mouse oocyte exists within the follicle when in the ovary, it is intimately associated with the surrounding follicle cells until after ovulation. This relatively new method allows for the microinjection to occur while the oocyte is cultured within the intact follicle under more physiologically relevant conditions. By microinjecting the follicle-enclosed oocytes, this group has been able to discover the mechanism(s) that maintain the immature oocyte arrested in meiosis and also to begin exploring the signaling mechanisms that are responsible for the reinitiation of meiosis (27–29). The technique is quite revolutionary and the concept of maintaining the proper physiological environment, as much as possible, is one that is applicable to all situations. In this chapter, you will also learn what type of music is most enjoyed by mouse oocytes.

In some cases, microinjection would be very useful but not considered because of the perceived difficulty of injecting sufficient numbers of cells for further analysis. **Chapter 13** describes a pressure-based method of injecting zygotes of the sea urchin *Paracentrotus lividus*. Using this method, up to several hundred embryos can be injected in a single session. In addition to the microinjection method, this chapter also describes experimental methods for perturbing gene function by either (1) producing and microinjecting synthetic mRNA produced in vitro, or (2) preparing linear DNA amplified by PCR for direct microinjection into the embryo. The advantages and disadvantages of these two different methods are discussed.

The final two chapters describe practical uses of the technique of microinjection. Methods for imaging human gametes and zygotes after intracytoplasmic sperm injection (ICSI) are presented in **Chapter 14**. The techniques focus on the identification of cytoskeletal elements and the role these components play during fertilization. Procedures are given in great detail for the removal of follicle cells and the zona pellucida, and fixation procedures optimized for fluorescence immunocytochemistry and for examination by conventional electron microscopy and ultrastructural immunolocalization.

Chapter 15 presents a method for somatic cell nuclear transfer (SCNT) in the mouse. This article directly addresses the fact

that cloning by SCNT has always been difficult and inefficient (30, 31). In the protocol described here, donor nuclei from cumulus cells are injected directly into mouse oocyte. While this has been accomplished before, the Kishigami and Wakayama protocol results in a twofold to fivefold improvement in embryo developmental rates by the inclusion of trichostatin A, a histone deacetylase inhibitor. The TSA may ‘reprogram’ the somatic cell nuclei, making them more amenable to the early developmental program. Also significant in **Chapter 15** is a description of the use of a piezo-actuated micromanipulator that allows the use of larger microinjection needle tips with less damage to the oocyte.

3. Conclusion

The methods described in this book should allow any lab to incorporate the technique of microinjection into their experimental repertoire. Whether DNA, RNA, or protein is the molecule of interest, microinjection provides a mean of studying function within the context of the living cell. The technology is remarkably accessible and relatively inexpensive, while the possibilities are virtually endless.

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