Video Article

Single Cell Transfection in Chick Embryos

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Abstract

A central theme in developmental biology is the diversification of lineages and the elucidation of underlying molecular mechanisms. This entails a thorough analysis of the fates of single cells under normal and experimental conditions. To this end, transfection methods that target single progenitors are a prerequisite. We describe here a technically straightforward method for transfecting single cells in chicken tissues *in-ovo*, allowing reliable lineage tracing as well as genetic manipulation. Specific tissue domains are targeted within the somite or neural tube, and DNA is injected directly into the epithelium of interest, resulting in sporadic transfection of single cells. Using reporters, clonal populations may consequently be traced for up to three days, and behavior of genetically manipulated clonal populations can be compared with that of controls. This method takes advantage of the accessibility of the chick embryo along with emerging tools for genetic manipulation. We compare and discuss its advantages over the widely-used electroporation method, and possible applications and use in additional *in-vivo* models are also suggested. We advocate the use of this method as a significant addition and complement for existing lineage tracing and genetic interference tools.

Video Link

The video component of this article can be found at https://www.jove.com/video/2133/

Protocol

1. Preparation of Micropipettes

We pull our pipettes on a standard Narishige pp-83 pipette puller with a heat setting of 13.5. The precise setting must be calibrated, aiming at a pipette tip diameter of between 8 and 10 microns. We use filament-containing borosilicate glass tubes with an outer diameter of 1.0 mm and inner diameter of 0.75-0.78 mm. The pipettes are consequently stored in a plastic dish with the tips protected from contact with the edges of the dish.

2. DNA Preparation

The plasmid of interest is transfected into DH5α *E. coli* using standard procedures. Overnight cultures from single colonies are used to purify the plasmid using maxi-prep kits (any commercial kit should do). We use a plasmid concentration of 1.0 µg/ µL for non- clonal injections. Higher concentrations increase clogging of pipette tips. For clonal applications, plasmid concentrations are much lower and must be adjusted (see below). For pCAGG-GFP, the optimal concentration producing clonal results was found to be about 0.05-0.1 µg/ µL. One to 2 µL of plasmid is typically used per experiment, and a minimal amount of fast-green powder (adhering to the tip of a dry, sharp glass pipette) is added to the plasmid before the experiment begins. The plasmid is then centrifuged in a tabletop centrifuge for 2 minutes at maximal speed. This procedure seems to reduce pipette-clogging problems.

3. Injection of Embryos

3.1 Preparation of embryos

We use quail as well as chicken eggs for injections. The eggs are incubated lying on their long axis until the desired developmental stage. The eggs are swabbed with 70% ethanol and allowed to dry. The pointed end of the egg is pierced with surgical scissors (this is necessary only in chicken eggs) and 1 mL (in quails) or 2 mL (chick) of albumen is drawn from the egg with a 10 mL syringe (19G needle). The holes are then sealed with hot paraffin.

Prior to actual injection, a window is opened at the top of the egg shell using surgical scissors. Placing sticky tape over the shell and cutting through it can help reduce debris from the shell. PBS buffer containing antibiotics is pipetted immediately into the egg. To access the embryo, the

vitelline and/or amniotic membranes are cut and diverted using 0.14 mm insect pins mounted onto a needle holder. Non-toxic ink (Pelican #17) is injected underneath the blastoderm to help visualize the embryo, using a fire-pulled capillary mounted on an aspirator.

3.2 Loading and mounting of micropipettes

The puller micropipettes can be loaded with the plasmid by fire-pulled capillaries of adequate diameter, mounted onto an aspirator. A minimal amount of plasmid is drawn using the capillary and loaded into the pipette from its opening opposite to the sharpened tip, and approaching the tip from the inside as much as possible. The pipette is consequently loaded onto a manual air pressure injector mounted on a micromanipulator. Minimal pressure is applied, if necessary, in order to allow the plasmid solution to reach the tip of the pipette.

3.3 Plasmid injection

The pipette is manipulated into the liquid medium of the egg while applying some air pressure to prevent clogging of the pipette tip. The relevant tissue domain is pierced. The change in pressure as the tip enters the tissue is often enough to release plasmid with visible fast green. If this is not the case, apply slight pressure to the syringe until the plasmid/fast green are ejected. This process can be repeated along the axis of the embryo, for example in adjacent somites or along the extent of the neural tube. Following injections, PBS may be added. The window is sealed using sticky tape (parafilm is not desirable especially for quail eggs-it increases embryo death rates, probably by blocking exchange of oxygen through the shell). The treated embryos are returned to an incubator. Avoid ventilation and place small containers with water in the incubator as they help maintain moisture and increase yields.

4. Adjusting Injection Parameters and Monitoring Success Rates

Asessing and calibrating various injection parameters is critical for applications aiming at single-cell analysis. Not every injection event will lead to successful labeling of a single cell. Plasmid concentration which is too high can lead to non-clonal labeling, while very low concentrations result in high failure rates. The concentration of the injected plasmid must be optimized for clonal injections. We recommend dilution of the entire purified plasmid stock rather than diluting individual preps to a pre-determined concentration. The latter might introduce some variance of success rates, probably due to variations in purity and other parameters such as the proportion of supercoiled plasmid.

4.1 Assessing success rates by whole mount observation

Initial assessment of injected embryos can be performed by whole-mount observation under a fluorescent binocular. Six to eight hours post-injection are sufficient to assess success rates with a pCAGG-enhanced GFP construct. This is provided the target site is superficial enough to be readily apparent (e.g. the dorsal somite, dorsal neural tube, ectoderm etc, see Figure 1A-C.). This step is particularly helpful for evaluating experimental series exhibiting either too many labeled cells or alternatively, lacking any fluorescent cells. If further incubation is desired, antibiotics-containing PBS solution should be added to the egg after viewing and the sealing tape should be replaced.

4.2 Assessing clonality by serial-section analysis

The best means for assessing the rates of successful injections is by serially-sectioning injected embryos and using immunohistochemistry to visualize the labeled cells. Under these conditions, incubation times can be as short as 4 hours post-injection. The embryos are fixed and processed for paraffin sectioning (or cryosectioning). Glass-mounted sections are then de-paraffinized and immuno-stained for reporter visualization. Amplification methods such as biotin-streptavidin might be desirable. Finally, the serial sections are scanned to detect labeled cells in injected segments. If upon initial trials multiple rather than single cells are obtained, the plasmid stock must be diluted and the process repeated until adequate results are achieved. Two to 10-fold dilutions of the plasmid stock solution may be necessary in cases of success rates being too high.

For each plasmid to be transfected, a thorough calibration that ensures clonality should be repeated.

5. Representative Results

We typically carry out a single injection of pCAGG-GFP per somite, while targeting 6-12 segments per embryo [1,2]. Under this setting, a clear signal can be detected by whole mount observation 8 hours post injection, in at least one segment of one embryo out of ten. Under these conditions, labeling was further confirmed to be clonal (Figure 1B,C). When using high DNA concentrations, most embryos may exhibit at least one successful injection, raising the possibility that multiple cells have been transfected.

Serial section analysis revealed that clonal transfections were attained when the success rate was about 10%. This means that in order to achieve clonality, the technique is per definition, highly inefficient. On the other hand, under these conditions, more than 93% of positive labeling events were found to be clonal [1].

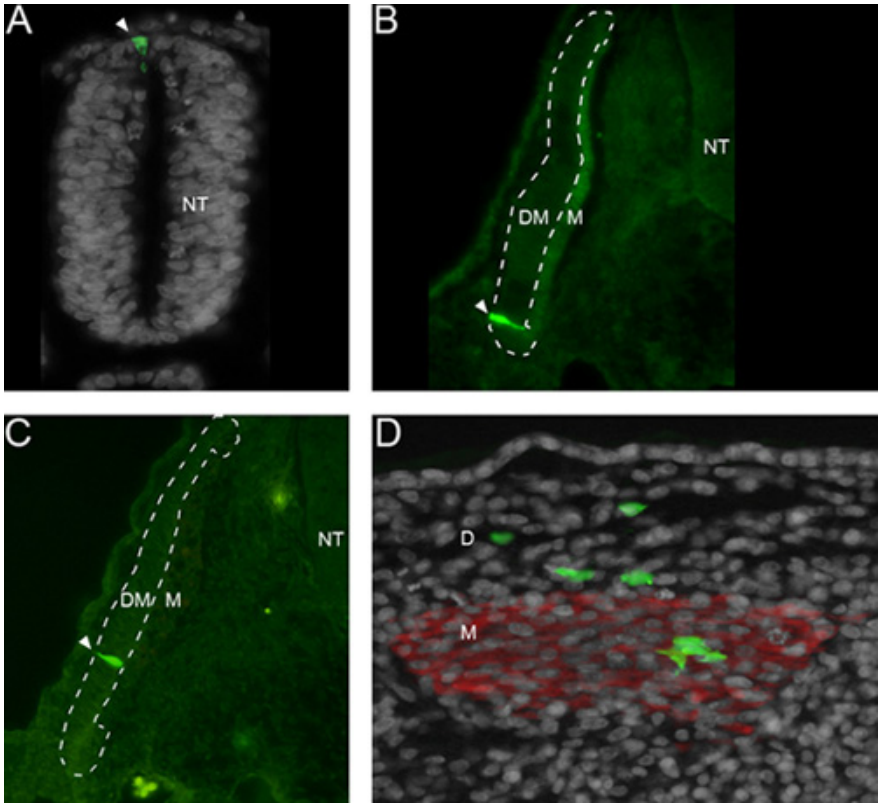

Figure 1. Clonal injections of pCAGGS-GFP into neural and skeletal progenitors. (A-C) Transverse sections showing labeling of the dorsal neural tube (NT) (A), ventrolateral domain of the dermomyotome (DM) (B), or central DM sheet (C), 6hr after microinjection of pCAGGS-GFP to a single progenitor. (D) Two days following clonal transfection of the central DM, single progenitors generated derivatives in both dermis (D) as well as muscle (M) (see ref.[1] for details).

Discussion

The method described above is a modification of a previously described technique for delivering lipophilic dyes to small cell subsets or single progenitors [3]. It has three main advantages over the standard electroporation technique. First, it provides a means for labeling with confidence discrete sites in target tissues, allowing much greater spatial resolution than electroporation (see for example Figure 1B,C). Second, it permits labeling of single cells, thus allowing to trace their lineage *in-vivo* under otherwise normal conditions [1 2] (Figure 1C,D). Third, gene miss-expression at the single-cell level provides a tool for investigating molecular mechanisms of lineage segregation, for studying the role of growth factors or of other genes on cellular behavior in an otherwise normal background, etc. Therefore, this method complements the widely applied electroporation technique, and further exploits the accessibility of the avian embryo to spatio-temporally regulated gain and loss of gene function *in-vivo*.

An increasingly relevant application is the analysis of cellular behaviors at the single cell level (cell migration, axonal guidance, cell division etc.) using emerging live imaging technologies [4 5 6 7]. In some of these experimental settings, it might be desirable to use a rapid-activation reporter species (such as Venus-GFP) [8] in order to allow an earlier visualization of the injected cell.

Given the simplicity of the method here described, it is very likely that it could also be applied to other animal models such as Zebrafish and *Xenopus* . The system is very easy to set up and use, and the main difficulty is generating sufficiently large series of labeled embryos, as well as scanning for labeled progeny in applications requiring serial-sections. In our hands, injected cells exhibited an easily detectable GFP signal for up to 3 days, but this parameter is likely to depend on rates of cell proliferation. Using a long-lived reporter such as Lac-Z, or transposon-mediated gene transfer leading to stable integration [9], it might be possible to prolong tracing of the progeny.

Disclosures

No conflicts of interest declared.

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