Microinjection of RNA into *Xenopus* oocytes

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**Abstract**

In this Userguide microinjection is used to study mRNA transport and localization in stage I - II *Xenopus* oocytes. During oogenesis in various animal models, maternal mRNAs and proteins are localized to specific regions of the oocyte. These macromolecules are maternal determinants, and they provide the basis for patterning in the developing embryo.

**Introduction**

Microinjecting RNA into oocytes and eggs is a very common method used to study gene expression during embryonic development [1]. In the *Xenopus* developing oocyte, over a dozen maternal mRNAs are localized during maturation [2]. To study the localization process, synthetic fluorescent mRNAs can be microinjected and their transport visualized by confocal microscopy.

Here, we describe a technique which is successfully used in our laboratory to study mRNA transport and localization in stage I - II *Xenopus* oocytes [3].

**Equipment**

- Dissection microscope (Olympus SZ61)
- Microinjector FemtoJet® express (Eppendorf, Germany) (Fig. 1)
- External pressure supply (Model 3, Jun-Air, USA)
- Micromanipulator MM33 (Märzhäuser, Germany)
- Microcapillaries Femtotip® (Eppendorf, Germany)
- Microcapillaries Harvard GC100T-10 (Harvard Apparatus LTD)
- Micropipette puller (PN-30, Narishige, Japan)
- Centrifuge (e.g. Centrifuge 5424, Eppendorf, Germany)
- Microloader (Eppendorf, Germany)
- Beveled microscope slide
- Oocyte holder (Fig. 2)

**Figure 1:** FemtoJet express microinjector

**Figure 2:** Oocyte holder (self construction), made of acrylic glass. See "Microinjection" section for details.
Materials

- Oocytes
  Adult or juvenile *Xenopus laevis* specimens were purchased from Xenopus Express (Plant City, USA) or CNRS (Rennes, France). Stage I - II oocytes (staged according to the method described in [4]) were released from dissected ovaries at various times after collagenase A treatment.
- Mineral oil (Sigma M8410, SIGMA-Aldrich, USA)
- DiI (DiIC16(3), Molecular Probes, USA) saturated solution in Wesson pure vegetable oil (soja) [5]
- Collagenase solution
  100 mM NaH$_2$PO$_4$ (pH 7.4) containing 1.33 mg/ml Collagenase 1A (Sigma C 9891, Sigma-Aldrich, USA) and ovoid trypsin inhibitor (10 mg/ml, Sigma T 2011, Sigma-Aldrich, USA) [6,7]
  We aliquot 15 x concentrated stocks, for convenience, of Collagenase 1A and trypsin inhibitor, and store them at –20 °C. A 15 ml final working volume solution is sufficient to process up to 5 ml of ovary.
- 50 % L-15 medium
  Leibowitz medium (Sigma L5520) supplemented with 1 mg/ml bovine serum albumin (BSA), 100 µg/ml gentamicin, 1 U/ml penicillin, and 1 µg/ml streptomycin [6].
- 50 % L-15 culture medium
  For culturing St I-II oocytes 50% L-15 medium (without BSA) is additionally supplemented with 1 mM L glutamine, 1 µg/ml insulin, 15 mM HEPES (pH 7.8), 50 U nystatin and 5% serum containing vitellogenin (VTG) [8]. For best results the culture medium should be changed daily.
- Alexa-488-5-UTP-labeled mRNA
  The probes are stored at –80 °C and centrifuged at g-max in a tabletop centrifuge (17,530 x g) for 25 min before use.

Methods

Obtaining oocytes

The dissected ovary lobes are teased open with forceps in the collagenase solution and incubated 15 min on a 60 rpm rotary shaker at 18-20 °C. Thereafter, at 5 min intervals, the ovary lobes are shaken with forceps in 50 % L-15. The stage I - II oocytes (Fig. 3) can be easily seen under a dissection microscope and are then transferred into fresh 50 % L-15 medium with a drawn-out pasteur pipette which has been flamed at the end to prevent oocyte damage.

Preparation of Alexa 488-5-UTP Fluorescent mRNA *
(modified after Glotzer et al, 1997) [9].

<table>
<thead>
<tr>
<th>For a final reaction volume of 25 µl:</th>
<th>µl</th>
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<tbody>
<tr>
<td>H$_2$O (as needed)</td>
<td>XX</td>
</tr>
<tr>
<td>5x Transcription buffer (Promega, USA)</td>
<td>5.0</td>
</tr>
<tr>
<td>DTT 100 mM (Promega, USA)</td>
<td>2.5</td>
</tr>
<tr>
<td>RNASin 40 U/µl (Promega, USA)</td>
<td>1.0</td>
</tr>
<tr>
<td>ATP*</td>
<td>0.5</td>
</tr>
<tr>
<td>CTP*</td>
<td>0.5</td>
</tr>
<tr>
<td>UTP*</td>
<td>0.4</td>
</tr>
<tr>
<td>GTP (stock aliquotes stored at –80 °C)</td>
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</tr>
<tr>
<td>GTP capped 40 mM (Promega, USA)</td>
<td>2.5</td>
</tr>
<tr>
<td>Alexa 488-5-UTP (Molecular Probes, USA)</td>
<td>5.0</td>
</tr>
<tr>
<td>DNA (linearized, 2 µg final)</td>
<td>XX</td>
</tr>
<tr>
<td>RNA polymerase 80 U/µl</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* NTPs 50 mM stock, stored at –20 °C (Sigma Aldrich, USA)

The reaction mix should be gently vortexed and spun, then incubated at 37 °C for 2-3 hours. The RNA is then purified with the following procedure.
RNA Purification

1) Add 2.0 µl RQ1 DNAse (Promega, USA), mix, spin, and incubate a further 15 min at 37 °C.
2) Add 11.2 µl 10 M LiCl, mix, add 120 µl ethanol tempered at -20 °C, mix, and incubate at -20 °C for 15 min.
3) Prepare two 1% agarose mini gels, one without ethidium bromide (EtBr) and the second with EtBr in 1x TAE buffer, set thermostat heating block to 60 °C.
4) Centrifuge at 4 °C and g-max (table top centrifuge) for 15 min.
5) Discard supernatant, add 200 µl 70% ethanol, and re-spin as in 3)
6) Discard supernatant, let pellet dry 5-10 min at room temperature and dissolve pellet in 20 µl DEPC H$_2$O.
7) Add 30 µl STE buffer (10 mM Tris pH 8.0, 1.0 mM EDTA, 150 mM NaCl), keep on ice.
8) Prepare G-50 micro columns (ProbeQuant G-50 Micro Columns, GE Healthcare, USA), vortex, snap off end, unscrew cap ¼ turn, place in microtube and spin 1,000 rpm (800 x g) for 1 min, discard tube and put column into a new tube.
9) Load the 50 µl sample onto column without disturbing the surface. Place the tube back into the centrifuge (if fixed angle rotor, then take care to position the column at the same gel angle as in the pre-spin) and spin for 2 min at 1,000 rpm (800 x g).
10) Discard column. Add 0.4 volume (20 µl) of 10 M LiCl and 120 µl ethanol (pre-cooled at -20 °C), after incubating at -20 °C for 15 min repeat steps 4) and 5)
11) Discard supernatant, let pellet dry 5-10 min at room temperature, and dissolve in 10 µl DEPC H$_2$O. Place tube on ice.
12) Prepare samples (including RNA markers (Promega, USA) for the gel containing EtBr) containing 5 µl loading buffer (Ambion, UK), 5 µl DEPC H$_2$O, 0.2 µl RNA.
13) Heat gel samples for 2 min at 60 °C and load immediately.
14) Run the 1% agarose mini-gels (RunOne Electrophoresis, Embi Tech, USA) at 100 V for 20-25 min in 1 x TAE buffer.
15) According to A260 results and gels, one can now adjust the concentration of the synthesized RNA (our working concentration is nominally 1 µg/µl)

For those who prefer using kits, we have (at least for our probes) optimized Alexa 488-5-UTP for mMessage mMachine (Ambion, UK) which also gives comparable results for a 20 µl final reaction volume:

<table>
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<th>µl</th>
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<tr>
<td>H$_2$O (as needed)</td>
<td>XX</td>
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<tr>
<td>2 x NTP</td>
<td>6.0</td>
</tr>
<tr>
<td>Alexa 488-5-UTP</td>
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</tr>
<tr>
<td>10 x Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>1 µg DNA</td>
<td>XX</td>
</tr>
<tr>
<td>10 x SP6 mix</td>
<td>2.0</td>
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<td></td>
<td>20</td>
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</table>

After 2-3 hours incubation at 37 °C, the above RNA purification procedure is used.

Annotations:
- Both RNA procedures are employed in our lab. Total yields are between 6-10 µg with SP6, although much higher yields are obtained with T7 and T3 promoters.
- Alexa-488-5-UTP labeled mRNA: the probes are stored at –80 °C, and are centrifuged at 17,500 x g, 4 °C in a tabletop centrifuge for 20-25 min before use. Take all necessary precautions to guard against RNase contamination. We have a separate mini-gel apparatus for RNA which is periodically treated with 0.1 N NaOH for 30 min, followed by washing with distilled water. All tubes and tips are autoclaved. Disposable gloves should be worn.
Preparation of DiI saturated solution

Several grains of DiI are deposited into a microtube (1.5 ml, Eppendorf, Germany), add 500 µl Wesson oil (our stock bottle is kept at 4 °C) and vortex several times. Then incubate the tube overnight at RT, vortex again and then spin at g-max (17,500 x g) for 15 min in a table top centrifuge. This solution is stable for about one year at 18-20 °C. Centrifuging before use is not necessary if the tube is kept upright. Keep the tube covered with aluminum foil.

Preparation and filling of the microcapillaries

The injection pipette tips are one of the crucial aspects of successful experiments. We check tip integrity, after pulling them, under the dissection microscope. Nominal settings for the Narishige PN-30 micropipette puller are the following parameters: heater 80, sub magnet 19 and main magnet 48. It is very important to use freshly made pipettes because they could become grungy within one or two days. After loading an injection pipette and mounting in the pipette-holder, the tip is broken on the beveled edge of the microscope slide, of which the bevel can easily be made by running the edge over a piece of 600-1000 grit sandpaper. The slide is treated with 0.1 N NaOH for 30 min, rinsed in DEPC H₂O, dried, and then kept in a 50 ml Falcon tube.

Beginners should, however, use Eppendorf Femtotips which will give them a good idea of optimal tip characteristics. In addition, they are manufactured with open-ended tips which preclude the extra breaking step. It is, however, possible to adjust the Narishige puller to achieve open-ended tips, but with many users, any slight change in filament shape or position will result in varied tip characteristics. It is recommended to backfill your injection pipettes with Eppendorf Microloaders. The advantage is their autoclavability but we have yet to experience probe degradation. With the pipettor set at about 1.5 µl, the Microloader tip is dipped into the centripetal part of the probe, away from any particles which might block up the injection tip. With practice, one can pipette up about 4 mm length of the Eppendorf Microloader which is sufficient to inject at least 50 oocytes with 100 pl of probe each. We suggest to keep the box of Microloaders in a separate plastic box with a lid, to help keep out airborne dust and other contaminants.

Microinjection

With our methodology, a nominal injection pressure of approx 28-30 psi (NB: higher pressure indicates smaller tip diameter and less mechanical damage to the oocyte plasma membrane) (injection time Ti: 0.5 sec, back pressure Pc : 0.5 psi) delivers 100 pl into the mineral oil (droplet size measures approximately 60 µm in diameter as measured with the ocular graticule).

The injection tray (Fig. 2) was milled first with a 4 mm diameter countersink drill to a depth of 1 mm into a 6 mm thick acrylic glass slab. A second 1.5 mm wide slot was then milled to a depth of 1.5 mm at one side of the first slot. The tray can be treated with 0.1 N NaOH for 30 min and then rinsed with fresh Milli-Q water. (Do not use ethanol or acetone, for these will deteriorate the acrylic glass.)

1) Transfer the oocytes into the injection tray’s 1.5 mm slot in 50 % L-15 medium.
2) Adjust the level of medium in the 1.5 mm slot for optimized viewing.
3) By slightly tilting and jiggling the tray, the oocytes will form a single line.
4) Move the needle towards the oocyte. Just before the needle enters the liquid, inject once to ensure that the resulting droplet is the correct size and that the needle has not become blocked.
5) Afterwards inject each oocyte, wait for a moment, and then withdraw the needle gently. Once the set of oocytes has been injected, add additional 50 % L-15 medium and the oocytes can then be removed with the mouth pipette.

We first inject DiI to stain for endoplasmic reticulum (ER) including the mitochondria cloud (MC), and then transfer the surviving oocytes into fresh 50 % L-15 medium. The DiI solution is injected with the “clean mode” (right mouse click) as normal injection pressures (i.e., 25 – 28 psi) are barely sufficient to inject the more viscous oil. After subsequent injection of Alexa 488 5-UTP labeled mRNA, the oocytes are then transferred to 50 % L-15 culture medium. Confocal imaging delineates the DiI-stained ER and the MC (Fig. 4A) and concomitantly the localization of Alexa 488 5-UTP labeled mRNA into the MC (Fig. 4B).
Fig 4 A,B: Confocal images of Dil-injected (A) and Alexa 488 5-UTP mRNA (B) St I oocytes. The mRNA has localized in the MCs 24h after injections. ER, endoplasmic reticulum; GV, germinal vesicle; MC, mitochondrial cloud. Scale Bar 100µm.

References


### Eppendorf Ordering information

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<td><strong>FemtoJet express</strong></td>
<td>5248 000.017</td>
<td>920010521</td>
</tr>
<tr>
<td>Programmable microinjector with external pressure supply</td>
<td></td>
<td></td>
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<tr>
<td><strong>Centrifuge 5424</strong></td>
<td>5424 000.410</td>
<td>022620401</td>
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<tr>
<td>Includes aerosol-tight 24 x 1.5/2 ml rotor and lid, with rotary knobs</td>
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<td><strong>Microloader</strong></td>
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<td>Pipette tip for filling the microinjection capillaries</td>
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<td><strong>Femtotip® II</strong></td>
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