Chapter 19

Studying Protein-Tyrosine Phosphatases in Zebrafish

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Abstract

Protein-tyrosine phosphatases (PTPs) are a large family of signal transduction regulators that have an essential role in normal development and physiology. Aberrant activation or inactivation of PTPs is at the basis of many human diseases. The zebrafish, *Danio rerio*, is being used extensively to model major aspects of development and disease as well as the mechanism of regeneration of limbs and vital organs, and most classical PTPs have been identified in zebrafish. Zebrafish is an excellent model system for biomedical research because the genome is sequenced, zebrafish produce a large number of offspring, the eggs develop outside the mother and are transparent, facilitating intravital imaging, and transgenesis and (site-directed) mutagenesis are feasible. Together, these traits make zebrafish amenable for the analysis of gene and protein function. In this chapter we cover three manipulations of zebrafish embryos that we have used to study the effects of PTPs in development, regeneration, and biochemistry. Microinjection at the one-cell stage is at the basis of many zebrafish experiments and is described first. This is followed by a description for measuring regeneration of the embryonic caudal fin, a powerful and robust physiological assay. Finally, the considerable but manageable troubleshooting of several complications associated with preparing zebrafish embryos for immunoblotting is explained. Overall, this chapter provides detailed protocols for manipulating zebrafish embryo samples with a compilation of tips collected through extensive experience from the zebrafish research community.

Key words Zebrafish, Protein-tyrosine phosphatases, PTP, Microinjection, Regeneration assay, Tissue lysis

1 Introduction

Protein-tyrosine phosphatases (PTPs) are a large family of signal transduction regulators, determining rate and duration of phosphotyrosine (pTyr) phosphorylation cascades [1]. Disruption of PTP activity leads to aberrant pTyr signaling and is at the basis of many human diseases [2]. Much is already known of the mechanisms PTPs employ to regulate signal transduction [3] and the regulation of their dephosphorylating activity [4–6]. Yet, a full appreciation of the importance of temporal and spatial control of PTP activity is best acquired in vivo. Model organisms used to acquire such insights include the invertebrates *Drosophila* [7] and *C. elegans* [8] and the vertebrates *Danio rerio* [9], mouse [10], and rat [11].

Rafael Pulido (ed.), *Protein Tyrosine Phosphatases: Methods and Protocols*, Methods in Molecular Biology, vol. 1447,
DOI 10.1007/978-1-4939-3746-2_19,

The zebrafish *Danio rerio* is being used extensively to model major aspects of development [12] and diseases including cancer [13, 14], metabolic disease [15–17], and cardiovascular disease [18]. Zebrafish are even used to study the mechanism of regeneration of limbs and vital organs [19, 20]. Evidently, zebrafish studies are a key aspect of translational research and are enabling advancements in human health [21]. Zebrafish are oviparous, provide up to 200 embryos per mating pair per week and develop within 5 days, with most organs forming within 48 h. Many existing genetic mutants are available from stock centers (Zebrafish International Resource Center in Eugene, USA, and European Zebrafish Resource Center in Karlsruhe, Germany) and mutagenesis is highly feasible using optimized transcription activator-like effector nucleases (TALENs) [22] or the combination of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with the CRISPR associated protein (CAS) [23–25]. Transient overexpression or stable transgenesis can readily be achieved through microinjection of synthetic messenger RNA (RNA) or plasmid DNA respectively at the one-cell stage [26]. In addition, mutagenesis-based gene inactivation is carried out using reagents such as *N*-ethyl-*N*-nitrosourea (ENU) to generate random single nucleotide mutations [27].

Zebrafish embryos are transparent, allowing for easy analysis of developmental progression and defects as well as disease phenotypes. Intravital imaging of fluorescent markers expressed in a tissue- or cell type-specific manner provides a powerful tool for studying developmental processes. This has been taken advantage of extensively to study angiogenesis [28], lymphangiogenesis [29], convergence and extension cell movements during development [30] and even to capture embryonic development in 3D [31, 32]. Intravital imaging of fusion proteins facilitates the analysis of protein localization, protein–protein interactions, and protein function in a whole organism in vivo. In addition, several techniques are now being refined and will augment the advantages of zebrafish as an experimental system. These include proteomics analysis of zebrafish embryos using mass-spectrometry following selection by immunoprecipitation [33–35], analysis of the plasma proteome following blood collection [36] and derivation of cell lines from single embryos [37].

Finally, the relative simplicity of adding compounds to either the water of the adults or the medium of the embryos, combined with the possibility for large sample number and rapid reproducibility has led to several bioactive compound screens [18, 38]. Notably, combining knockouts or embryos with transient overexpression with timed drug treatments provides an unparalleled opportunity to pinpoint the exact temporal role of proteins in vivo.

An important experimental consideration is that the zebrafish genome was duplicated early in evolution [39, 40]. Whilst some of

the duplicated chromosomes were lost, the duplicated genes that remain may have complementary or diverging expression patterns and exhibit redundant or complementary functions. Therefore, gene duplications may complicate the creation of knockout mutants by requiring two redundant genes to be targeted. The translational value is another essential consideration when choosing an experimental system and the reason the zebrafish is regularly used to model disease is in part due to 84% of human genes associated with a disease having a zebrafish counterpart [41].

All classical PTPs, except *ptpn7*, *ptpn12*, and *ptpn14*, have been identified in the zebrafish genome and 14 are duplicated [42]. Van Eekelen et al. 2010 also characterize expression duration and localization using in situ hybridization, identifying that some of the duplicated PTP pairs have divergent expression patterns, indicative of diverging functions. A good example of the complexity arisen from PTP duplication is that of *ptpn11*, encoding the Shp2 protein. Bonetti et al. 2014 demonstrate that zebrafish *ptpn11a* and *ptpn11b* encode highly homologous proteins, Shp2a and Shp2b respectively. Yet, whilst *ptpn11a–/–* mutants have severe developmental defects and are embryonic lethal, *ptpn11b–/–* mutants show no obvious developmental defects. This difference may suggest that Shp2a and Shp2b proteins are functionally distinct. Shp2b does have a function in development, because *ptpn11a–/– ptpn11b–/–* mutants exhibit a slightly more severe phenotype than *ptpn11a–/–* mutants. Furthermore, severe developmental defects displayed by *ptpn11a–/– ptpn11b–/–* double mutant embryos are rescued by transient overexpression of either Shp2a or Shp2b, demonstrating functional similarity of Shp2a and Shp2b proteins. The expression patterns of *ptpn11a* and *ptpn11b* are distinct; *ptpn11a* is constitutively expressed at a high level throughout development, whereas *ptpn11b* expression is at a relatively low level during early development and increases steadily through later stages. Hence, we conclude that the difference in expression patterns of *ptpn11a* and *ptpn11b*, rather than an intrinsic difference in protein function of Shp2a and Shp2b is at the basis of the difference in function of *ptpn11a* and *ptpn11b* during development [43]. The accompanied complexity of gene duplication can be used to extensively delineate gene function and the advantages of zebrafish as an experimental system make it ideal for elucidating the intricate function and regulation of PTPs in vivo. Hence, zebrafish are often used to understand the role of PTPs in signaling in development [44–47] and, lately more, in disease [34].

2 Materials

All solutions are prepared in double-distilled, deionized Milli-Q filtered water (resistivity of 18 MΩ cm at 25 °C).

2.1 Microinjection

1. Bright-field/Nomarski optics stereomicroscope.
2. Micromanipulator.
3. Pneumatic microinjector.
4. Nitrogen (N_2) gas.
5. Glass capillaries: Outer diameter 1 mm, inner diameter 0.78 mm, length 100 mm.
6. 0.01 mm micrometer slide.
7. 10 μl microloader pipette tips.
8. 0.5% phenol red.
9. Thermomixer.
10. E3 medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$.
11. 0.05% methylene blue; dilute in E3 medium to 0.01% prior to use.
12. Micropipette puller P97.
13. Putty or tape.
14. Slanted lane mold.
15. Plastic 15 and 10 cm plates.
16. Ultrapure agarose.
17. 70% ethanol.
18. Mineral oil.

2.2 Regeneration Assay

1. Stereomicroscope.
2. Bright-field/Nomarski optics stereomicroscope with camera function.
3. 0.4% MS222: 0.4% ethyl 3-aminobenzoate methanesulfonate salt (MS222), 50 mM Tris–HCl pH 7.5; dilute in E3 medium to desired concentration prior to use.
4. Stainless steel surgical blade.
5. Plastic and glass Pasteur pipettes.
6. Plastic 10 cm dish and multi-well plates.
7. 10 μl microloader pipette tips.
8. E3 medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$.

2.3 Tissue Lysis for Protein Extraction

1. 1.5 ml tubes.
2. Mini-pestle (to fit in the 1.5 ml tubes).
3. 1 ml syringe and 0.2–0.8 mm needles.
4. Sonicator.
5. Degassed lysis buffer: 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 150 mM

NaCl, 0.25% deoxycholate, 1% Triton X-100, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol.

6. RIPA buffer: 25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate.
7. Ginzburg fish Ringers buffer: 111.2 mM NaCl, 3.35 mM KCl, 2.38 mM $NaHCO_3$.
8. Liquid nitrogen (N_2).
9. Thermomixer.
10. Cold centrifuge.
11. Protease inhibitors, aprotinin and leupeptin (both 1 mg/ml).
12. Phosphatase inhibitors sodium fluoride (0.5 M), beta-glycerophosphate (1 M), and sodium orthovanadate (200 mM).
13. 70% ethanol.
14. 2× Laemmli sample buffer: 2% β-mercaptoethanol, 20% glycerol, 0.125 M Tris–HCl pH 6.8, 4% sodium dodecyl sulfate, (a pinch) bromophenol blue.

3 Methods

The underlying principle of the protocols and notes that follow is that zebrafish embryos are a living organism, useful for analyzing phenotypes, but can also be considered a compact factory of cells malleable to existing molecular techniques. All procedures involving zebrafish described were approved by the local animal experiments committee and performed according to local guidelines and policies in compliance with national and European law. Zebrafish maintenance and breeding were performed following published protocols [48].

An absolute must for the genetic manipulation of zebrafish is the skill to microinject at the one-cell stage. This is covered first (Subheading 3.1) and following this a plethora of manipulations are available. Here, a description for measuring regeneration of the embryonic caudal fin, a powerful and robust physiological assay, is given (Subheading 3.2). Also the method of lysing zebrafish tissue for protein extraction to perform SDS-PAGE and immunoblotting will be detailed (Subheading 3.3), which requires considerable but manageable troubleshooting due to the complications that arise from obtaining a homogenous zebrafish cell lysate and lack of antibodies that recognize the zebrafish ortholog.

In Subheading 3.1 we cover the use of microinjection for introduction of alien genetic material into an organism, which began in 1971 when it was used to introduce DNA into Xenopus oocytes [49]. Since then the technique has been adapted for microinjection of zebrafish embryos at the one-cell stage and early mouse blastocysts for transgenesis [50, 51] and cells in culture [52]. A detailed

video protocol for microinjection of zebrafish embryos can be found on Jove entitled "Microinjection of zebrafish embryos to analyse gene function" [53]. Needles for holding and injecting RNA or DNA are made using a glass capillary micropipette puller, which contains a heating filament that slowly melts the glass and as the two halves separate the tip is stretched to the correct diameter. Each machine model will need to be calibrated according to the manufacturer's instructions before use.

RNA microinjection leads to efficient, and usually high, expression within several hours and will be described below, though the reader can consider all uses of "RNA" interchangeable with "DNA." The only significant factor when microinjecting DNA is that accuracy needs to be high to ensure that the DNA ends up in the cell. For this reason, significant effort is made to align embryos properly in the microinjection plate to improve accuracy of injecting directly into the cell. Microinjection is a technique that develops and improves with experience. It is easy to learn but difficult to teach as a lot is based on a singular coordinated movement of fingers moving the needle into the embryo and feet ejecting the RNA or DNA, and this is a "comfortable" feeling once mastered. For example, it takes practice to be able to estimate just how much of the needle needs to be shortened once mounted; there is no way to measure this. Practice is essential, and once the technique is acquired the individual tends to improve without any further guidance.

In Subheading 3.2 an embryonic caudal fin regeneration assay is described. Some urodeles and teleosts are capable of epimorphic regeneration, perfect or near-perfect replacement of lost tissue, throughout their lifetime [54]. Zebrafish are capable of regenerating multiple tissues, including fins, the brain, retina, spinal cord, and heart [19]. Zebrafish are therefore an excellent model to study and understand the mechanism of epimorphic regeneration. The results that emerge from such research may pave the way to enabling adult mammal organs to regenerate, most of which are currently limited to inflammation and formation of a collagen-rich connective tissue scar following injury [55]. Adult zebrafish caudal fin regeneration takes 10–12 days to complete. In comparison, embryo caudal fins only take 3 days, making the embryonic regeneration assay an efficient and reproducible technique. A previously established caudal fin regeneration model [56] was adapted to amputate at 2 days post fertilization and regeneration is then completed by 3 days post amputation (5 days post fertilization).

Importantly, as regeneration rate is higher in embryos, wound healing is also more rapid. For this reason it is paramount that a picture of the amputated caudal fin be taken as soon as possible to capture the wound margin as accurately as possible. Another consideration with an embryonic caudal fin regeneration assay is that changes in regeneration rate could also be due to changes in rate of embryonic development. It is ideal to compare amputated

embryos with same stage uncut embryos and, therefore a picture of an uncut embryo is taken at the same time as the embryo with the wound margin. This technique can easily be combined with microinjection of RNA or DNA or drug treatments.

Subheading 3.3 describes tissue lysis of whole zebrafish embryos for extraction of protein. Zebrafish embryos, up to 6 days, can be considered tissue extracts for applying molecular techniques. Thus, the method remains the same regardless of age but becomes more laborious with older, more defined tissue. Preparation of zebrafish embryos for SDS-PAGE requires deyolking, and then lysis buffer is applied before the sample is homogenized. A general method can be found on the ZFIN database [57] but we have developed our own lysis buffers and homogenization techniques for specific uses of the protein extract and this will be explained in detail below.

Zebrafish embryos have a yolk sac, providing nutrients for growth until day 6 when the embryos can eat particle food [48]. This yolk sac contains a high concentration of vitellogenin, a phospholipo-glycoprotein composed of multiple subunits, the most predominant two at around 150 and 80 kDa. Unfortunately, the yolk proteins interfere with detection of specific proteins by immunoblotting, presumably due to the high expression levels of the yolk proteins. Particularly detection of specific proteins of similar sizes as the yolk proteins is problematic. This interference can be largely reduced by deyolking embryos using Ginzburg fish Ringers solution [58]. After deyolking, lysis buffer is applied. We use one of two lysis buffers, each has advantages and disadvantages. Whereas RIPA lysis buffer is fast and provides high yield, the presence of SDS may disrupt delicate protein-protein interactions. In comparison, degassed lysis buffer will yield less protein but the absence of SDS protects delicate protein-protein interactions, which is useful if intending to perform co-immunoprecipitation. Following the choice of lysis buffer there are three options for homogenization. The least challenging, but also the least effective in terms of yield, is using a mini-pestle to crush the embryo in the 1.5 ml tube containing lysis buffer. A higher yield can be obtained by using a syringe and needle to aspirate and push the tissue through a small (<0.4 mm) needle opening (shearing). Complications arise with larger tissue (e.g., fin clips and embryos approaching 5 days post fertilization) which may clog the needle. Though laborious, this complication can be resolved by first choosing an appropriately sized (>0.6 mm) needle for the dense tissue extract and repeating homogenization with a smaller needle afterwards. The final alternative is to use a sonicator, which works well on embryos up to 4 days old but may take considerably longer on defined tissue such as fin-clips.

Following successful lysis of zebrafish tissue, the lysate can be loaded onto an SDS-PAGE gel following a standard protocol.

The only exception is that a protein concentration assay (e.g., Biorad Bradford assay) is not performed on zebrafish embryos because the sample is less pure than from cells and there is an abundance of yolk protein which will skew the estimation. Instead protein level is correlated with the number of embryos per unit volume of lysis buffer used. A standard protocol for immunoblotting can also be applied. A far greater problem is the sheer lack of antibodies that recognize the zebrafish protein homologs. It is not uncommon that when the zebrafish and mammalian homologs share a high percentage amino acid sequence similarity, the antibody raised against the mammalian homolog does not bind the zebrafish homolog. Even when the epitope sequence is conserved the antibody does not necessarily work for the zebrafish homolog; sometimes the antibody will simply not bind anything but more often the problem is increased nonspecific binding (including sequestration of the antibody by the yolk protein). This antibody issue may be partially circumvented by raising an antibody against the purified target zebrafish protein. However, functionality of these antibodies is not necessarily guaranteed and should be tested thoroughly for validation. Also of note is that these antibodies do not always have the same efficiency on endogenous proteins in zebrafish embryos. Hopefully, with time, a range of antibodies produced and validated by different labs will become available on the commercial market. Some are already available at the Zebrafish International Resource Centre (ZIRC).

3.1 Microinjection

1. Dissolve ultrapure agarose in E3 medium with 0.01% methylene blue to prepare a 2% agarose solution.
2. Pour 2% agarose into a plastic 10 cm dish and gradually apply the plastic lane mold onto the surface of the agarose. Leave new microinjection plate (Fig. 1) to set (*see* **Note 1**).

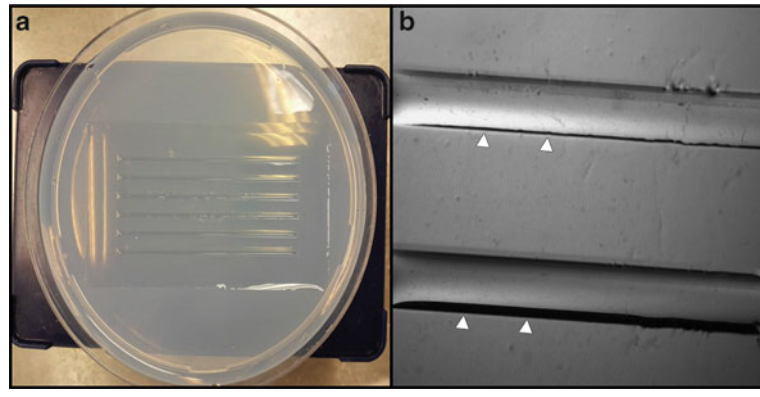

Fig. 1 Example of a microinjection plate made of 2% agarose (**a**) and higher magnification showing the grooves with the beveled edge (*arrow heads*) on the bottom side (**b**)

3. Remove the mold and pour E3 medium with 0.01% methylene blue to cover the surface. Store at 4 °C (*see* **Note 2**).
4. Prepare RNA by diluting as required in a mixture of Milli-Q water and 0.5% phenol red (*see* **Note 3**).
5. Heat RNA for 5–10 min at 65 °C and 1150 rpm in a thermomixer (*see* **Note 4**). Keep RNA on ice.
6. Switch on pneumatic microinjector and open the N_2 gas valve. The "regulator" pressure should not exceed 30 psi.
7. Wipe base of stereomicroscope with 70% ethanol and adjust lamp brightness as desired.
8. Place needle under stereomicroscope and use the micrometer slide to determine the point at which the needle tip width is equal to 20 μm. Use tweezers to remove the excess of the needle that extends beyond this point.
9. Use microloader pipette tips to transfer 1–2 μl of RNA into the cut needle.
10. Position micromanipulator and mount needle half way up the shaft of the pressure dispenser.
11. Position needle tip in a small dish of mineral oil and check pneumatic microinjector is set to "gated".
12. In "gated" mode apply pressure to the foot pump for 1–2 s to eject some RNA solution (*see* **Note 5**).
13. Switch pneumatic microinjector to "timed," switch the "range" to "100 ms" and the "period" to maximum "2.0" so that duration of ejection is 200 ms. Now, a single push of the foot pump will eject a single drop of RNA.
14. Press down on foot pump to eject a single drop of RNA into the mineral oil. Use the micrometer slide to measure size of droplet (*see* **Note 6**).
15. Adjust droplet size to 1 nl by first reducing the duration of ejection. Lower the "period" slightly and repeat **step 15**. If droplet size is still too small when duration of ejection is 70 ms ("period" is "0.70"), use the tweezers to remove more of the needle tip and repeat adjustment with "period" (*see* **Note 7**). Once a droplet of 1 nl is obtained rest the needle in mineral oil.
16. Collect fresh fertilized embryos in a plastic 10 cm dish with E3 medium. From this point on you have ~30 min before the single cell of the embryo divides. Work fast (*see* **Note 8**).
17. Transfer embryos to the lanes in the microinjection plate using a plastic Pasteur pipette (*see* **Note 9**).
18. Under the stereomicroscope the single cell of the embryo will be clearly visible. Position embryo such that the cell is at the top (*see* **Note 10**).

19. Use micromanipulator to adjust needle position and penetrate through the chorion and into the cell of the embryo. Almost simultaneously, press the foot pump to eject some RNA into the cell and withdraw the needle (*see* **Note 11**).
20. Move to the next embryo and repeat the microinjection until the desired number of embryos has been microinjected.
21. When finished use a plastic Pasteur pipette and E3 medium with 0.01% methylene blue to rinse the embryos into a plastic 10 cm dish with fresh E3 medium with 0.01% methylene blue. Incubate at 28.5 °C (*see* **Note 12**).
22. Discard needle in sharps bin and remaining embryos in designated waste bin. Close the N_2 gas valve, switch pneumatic microinjector to "gated" again and use foot pump to eject remaining N_2 gas. Switch off pneumatic microinjector and stereomicroscope light.

If injecting RNA encoding a fluorescent protein, a standard fluorescence microscope with appropriate filter can be used to visually assess success rate. It is common for protein expression to vary between embryos (Fig. 2) as each embryo is microinjected in a slightly different position and embryos have varying rates of protein synthesis, depending on health and developmental stage. Practice microinjections using mRNA encoding Green Fluorescent Protein (GFP) is convenient for evaluation of success rate. The technique is considered "mastered" once a success rate of 95% or higher is reached.

RNA or DNA injection into the cell at the one-cell stage of zebrafish should lead to expression in all subsequent daughter cells, but mosaicism is frequently observed. If RNA is microinjected at a late one-cell stage or whilst the first cell division is in progress

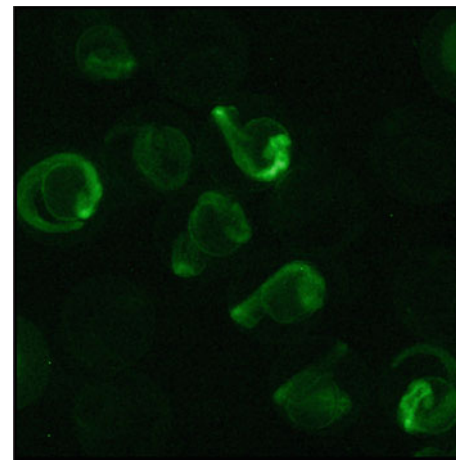

Fig. 2 Twenty-four hour post fertilization zebrafish embryos, still in their chorion, displaying variation in GFP protein expression between embryos following microinjection of GFP RNA

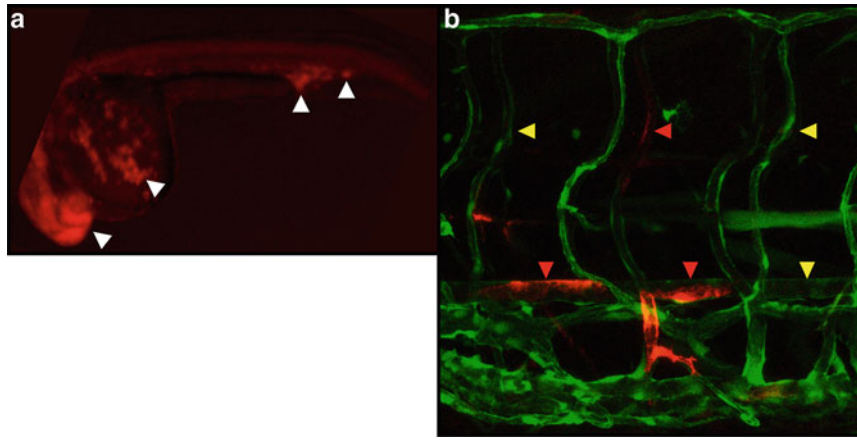

Fig. 3 Examples of non-ubiquitous expression following microinjection of RNA. (**a**) Example of patchy expression from RNA microinjection. An embryo microinjected with RNA encoding wild-type human Pten tagged with mCherry is imaged at 2dpf. Mosaic mCherry expression is found in a few clusters of cells scattered around the body of the embryo (*arrow heads*), suggesting RNA was not microinjected at the one-cell stage. (**b**) Example of mosaic expression in the trunk of an embryo at 5 days post fertilization. A transgenic embryo expressing *flt4:mCit* to mark the vasculature, shown in *green*, is microinjected with plasmid DNA encoding *prox1a:KalTA4,UAS:tagRFP,* shown in *red*, at the one-cell stage. Despite accurate microinjection only some vessels express RFP (*red arrow heads*), demonstrating mosaicism. *Yellow arrow heads* show similar vessels without RFP expression for comparison

mosaicism in the form of patchy expression, with only small clusters of cells translating the RNA, may arise (Fig. 3a). When RNA is microinjected accurately there may be considerably less mosaicism but it will still be present (Fig. 3b). Depending on the purpose of the experiment, the presence of mosaicism can offer some advantages in that a comparison can be made, for example, between the same population of cells expressing the microinjected protein and those without in the same embryo.

3.2 Regeneration Assay

1. Dechorionate embryos using tweezers.
2. Anesthetize embryos by transferring to 0.1% MS222 in E3 medium with 0.01% methylene blue for 2–4 min (*see* **Note 13**).
3. Place the lid of a 10 cm plastic dish over the light source of a stereomicroscope (*see* **Note 14**).
4. Transfer one embryo at a time to surface of plastic lid in a droplet of 0.1% MS222 in E3 medium with 0.01% methylene blue using a plastic Pasteur pipette (*see* **Note 15**).
5. Use a stainless steel surgical blade to amputate the caudal fin of the embryo immediately distal to the notochord under the stereomicroscope (*see* **Note 16**).
6. Use a glass Pasteur pipette to transfer embryo to fresh 0.1% MS222 in E3 medium with 0.01% methylene blue (*see* **Note 17**).

7. Capture wound margin of amputated caudal fin using a stereomicroscope with an attached camera (*see* **Note 18**).
8. Wash embryo in fresh E3 medium.
9. Use a glass Pasteur pipette to transfer embryo to a well in a multi-well plate containing fresh E3 medium with 0.01% methylene blue (*see* **Note 19**).
10. Repeat **steps 4–9** until multi-well plates of both amputated and uncut controls are filled. Incubate embryos at 28.5 °C for 3 days (*see* **Note 20**).
11. Prepare 0.2% MS222 in E3 medium by mixing half volume 0.4% MS222 and half volume E3 medium. Use a plastic Pasteur pipette to add three to four droplets of 0.2% MS222 in E3 medium to each well.
12. Use a glass Pasteur pipette to transfer one embryo per time to a 10 cm plate containing fresh 0.1% MS222 in E3 medium with 0.01% methylene blue.
13. Capture size of caudal fin using a stereomicroscope with an attached camera (*see* **Note 21**).
14. Repeat **steps 12–13** until all embryos of multi-well plates for both amputated and uncut controls have been imaged.

The images captured at 2 and 5 days post fertilization are of uncut controls and amputated embryos (Fig. 4). The change in

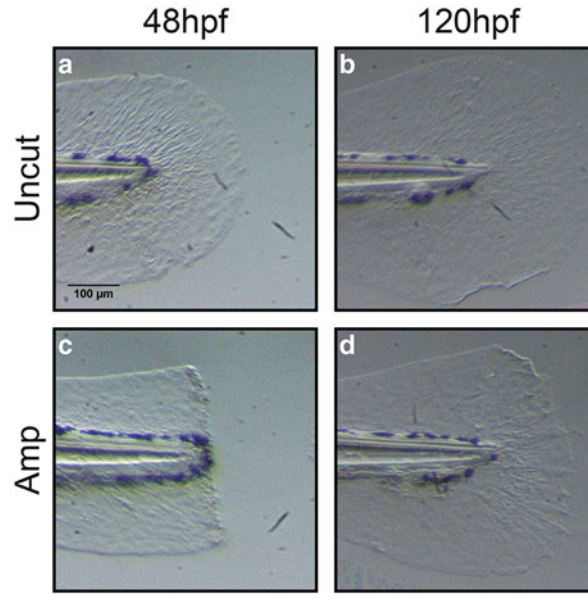

Fig. 4 Bright-field images of the caudal fin of zebrafish embryos. Fin margins are captured at 48 h post fertilization (**a** and **c**), and again at 5 days post fertilization (**b** and **d**), of uncut controls (**a** and **b**) and embryos amputated adjacent to the notochord (**c** and **d**). Scale bar is equivalent for all panels

caudal fin length after 3 days is calculated by subtracting the length of the fin from the tip of the notochord at day 5 from that at day 2 for each embryo. This is easily quantified using the measure tool in ImageJ and the difference calculated is a measure of regenerative outgrowth.

3.3 Tissue Lysis for Protein Extraction

1. Dechorionate embryos using tweezers (*see* **Note 22**).
2. Transfer embryos to a 1.5 ml tube, 96-well plate or similar. Remove as much system water or E3 medium as possible.
3. Prepare Ginzburg fish Ringers solution by adding protease and phosphatase inhibitors (10 μl/ml sodium fluoride, 5 μl/ml beta-glycerophosphate, 5 μl/ml sodium orthovanadate, 1 μl/ml aprotinin, 1 μl/ml leupeptin) and add sufficient volume to the embryos to allow repeated pipetting with a P200 tip (~600 μm wide).
4. Repeatedly pipette up and down in a gentle steady motion or invert regularly. Do not create air bubbles. Alternatively, place multiple embryos in a well of a 6-well plate filled with Ginzburg fish Ringers solution and place plate on a thermomixer set to 28.5 °C and 400–800 rpm for 30–45 min (*see* **Note 23**).
5. Wash deyolked embryos with fresh Ginzburg fish Ringers solution and transfer to chosen vessel (1.5 ml tube or 96-well plate).
6. Centrifuge briefly at 4 °C to collect embryos at bottom of tube or well.
7. Aspirate Ginzburg fish Ringers solution and snap-freeze embryos in liquid nitrogen (*see* **Note 24**).
8. Defrost frozen tissue on ice.
9. Prepare chosen lysis buffer by adding protease and phosphatase inhibitors (10 μl/ml Sodium Fluoride, 5 μl/ml beta-Glycerophosphate, 5 μl/ml Sodium Orthovanadate, 1 μl/ml Aprotinin, 1 μl/ml Leupeptin) and add volume according to age (Table 1).

Next, there are three homogenization options available for proceeding with tissue lysis; the steps required for homogenization using a mini-pestle (A), a syringe and needle (B), or sonication (C), are described below.

A. Mini-pestle homogenization (1.5 ml tubes only):

   10A. Quickly, with the tissue still frozen, start applying pressure using a mini-pestle that fits in a 1.5 ml tube (*see* **Note 25**).

   11A. Withdraw pestle and use a pipette to wash head of pestle to maximize amount of lysate collected.

12A. Clean pestle by rinsing with 70% ethanol followed by water and repeat **steps 10** and **11** for each sample.

13A. Rest on ice.

B. Syringe and needle homogenization:

10B. Wait for tissue to defrost in lysis buffer.

11B. Attach a sterile needle, width 0.2–0.8 mm, to a 1 ml syringe. No larger than 0.2 mm is highly recommended for 24 h post fertilization (hpf).

12B. Aspirate and dispense suspension repeatedly until tissue has been completely sheared (see Note 26).

13B. Rest on ice.

C. Sonication homogenization:

10C. Wait for tissue to defrost in lysis buffer.

11C. Incubate on ice for 30 min.

12C. Sonicate at "high" intensity for 10–15 min with a repeated cycle of 30 s ON, followed by 30 s OFF.

13C. Rest on ice.

14. Once homogenized with the chosen method centrifuge samples at 17,970 rcf and 4 °C for 20 min.

15. Transfer supernatant to a fresh tube or plate and discard the pellet. Can store supernatant at −80 °C at this point if required.

16. Mix equal volume lysate and 2× Laemmli buffer.

17. Boil samples for 5–10 min (*see* **Note 27**).

18. Store at −20 °C or continue loading samples onto an SDS gel.

Please note that there are many variations on the deyolking and homogenization procedures. For example, deyolking can be carried out *after* mini-pestle homogenization by centrifuging lysate and resuspending the pellet in Ginzburg fish Ringers solution. The sample is then centrifuged again to remove the Ginzburg fish Ringers

Table 1
Volume of lysis buffer to be used per embryo of the indicated age

Tissue age	Volume lysis buffer
<24 hpf	1 µl/embryo
24–48 hpf	1–2 µl/embryo
48–96 hpf	2–5 µl/embryo
>96 hpf	>5 µl/embryo
Adult fin clips	>10 µl

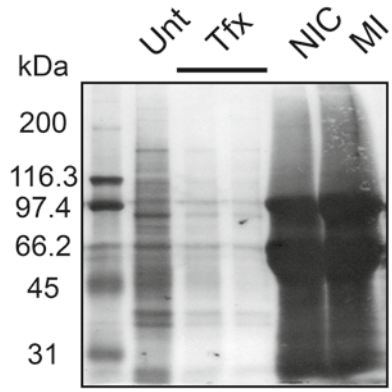

Fig. 5 Coomassie stain of PVDF membrane showing differences between HEK 293T cells lysed with RIPA buffer (*lanes 2–4*) and zebrafish embryos lysed with RIPA buffer and subjected to mini-pestle homogenization (*lanes 5* and *6*). A typical experiment comparing non-transfected (Unt) and transfected (Tfx) HEK 293T cells with non-injected control (NIC) and microinjected (MI) zebrafish embryo samples (7.5 embryo equivalents per lane). *Lane 1* contains a molecular weight marker

solution and resuspended in 1× sample buffer (created by mixing equal volumes of chosen lysis buffer and 2× Laemmli buffer). This way the deyolking procedure is more convenient but a possible consequence is greater variation in protein concentration between samples as some lysed protein may have been lost in the multiple centrifugations where supernatants are discarded. Another alternative is to only disrupt the integrity of the embryos a little using mini-pestle homogenization and follow that with sonication to achieve more efficient lysis. One's choice will be determined by a combination of purpose for the lysate, embryo age, and, ultimately, experience.

Preparation of zebrafish embryo lysates requires more diligence than for cell lysates. This is partly due to the high protein content compared to cultured cells, resulting in the bands being "fatter" and more "smiley" (Fig. 5). This can also cause protein bands to run slightly slower, but offsets size estimation by no more than 5 kDa. The right combination of deyolking, lysis buffer and homogenization optimizes the result. For example, embryos can be lysed in RIPA buffer prepared for SDS-PAGE using mini-pestle homogenization without dechorionating and deyolking (Fig. 6a). But when embryos are dechorionated, deyolked, lysed using degassed lysis buffer and prepared for SDS-PAGE using mini-pestle homogenization the result is less yield but almost as clean as that obtained from pure cell lysate (Fig. 6b). Then again, Fig. 6b clearly shows that deyolking can lead to unequal protein concentration between samples, as a result of both the lysate that remains on the head of the pestle and the centrifugation step required.

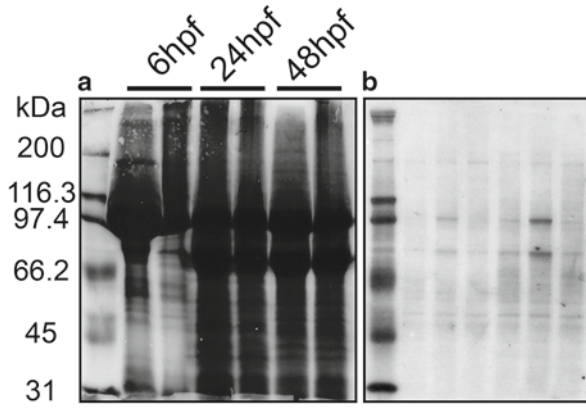

Fig. 6 Coomassie stains of PVDF membranes containing zebrafish embryo samples. (**a**) Samples, not dechorionated or deyolked, lysed in RIPA buffer and subjected to mini-pestle homogenization (5 embryo equivalents per lane). Zebrafish embryos were lysed at 6, 24, and 48 h post fertilization (hpf). Notice how "fat" and "smiley" the bands are. (**b**) Samples after dechorionating, deyolking, lysed in degassed lysis buffer and subjected to mini-pestle homogenization (1 embryo per lane). Zebrafish embryos were lysed at 48 hpf. Notice that the protein content is considerably less, but that the lysate profile is more "clean" and similar to that of the cell lysates seen in Fig. 5. A typical experiment with six different conditions is depicted. *Lane 1* in (**a**) and (**b**) contains a molecular weight marker

4 Notes

1. The plastic mold is embossed with ramps with a 45° beveled side (Fig. 1b). This aids microinjection as the embryo will be pushed into the corner of the lane, allowing the needle to more easily penetrate the chorion and yolk or cell.
2. The agarose microinjection plates will keep for 1–2 weeks, after which molds will begin to grow and the borders of the lanes begin to soften and break. The molds do not interfere with the embryos as long as the embryos are washed afterwards but the breaks in the lanes can inconvenience injections.
3. In general not more than 500 pg of RNA is injected in zebrafish embryos (this is achieved using 1 nl of RNA at 500 ng/μl). The phenol red is only used as a dye to observe successful injection (once competent this aid is no longer required), and 1 μl for every 15 μl is more than sufficient.
4. Heating the RNA removes secondary structures such as hairpin loops. This ensures that most of the RNA injected is available for translation and ultimately increases the efficiency of microinjection. This step is not necessary if injecting DNA.
5. This action removes air bubbles from the RNA. The RNA is now under pressure. If "hold" pressure is too great the RNA will now begin to leak from the needle tip, adjust if necessary.

6. Calibration of the RNA droplet to 1 nl is measured using a 0.01 mm micrometer slide. Briefly, according to $V=4/3\pi r^3$, a droplet of 1 nl has a radius of 62 μm and the diameter is hence 124 μm. The micrometer slide has divisions of 10 μm; meaning 1 nl is equivalent to 12 divisions. The needle width is measured to approximately 20 μm wide prior to mounting and the width of each new needle then needs to be adjusted to create a 1 nl droplet using the micrometer slide to calibrate.
7. Duration of ejection MUST NOT be <70 ms ("period" not <0.70) as this results in ejection of unreliable droplet size with repeated injections. If needle tip is shortened too much, a new needle will have to be prepared. Resting the needle tip in mineral oil avoids RNA drying up and forming a plaque that blocks the ejection of RNA. If, despite best efforts, the needle does become blocked there are two options; switch the pneumatic pump back to "gated" and try to flush the plaque off, or if the duration of ejection is still set relatively high (>100 ms for example) remove the tip of the needle with the tweezers and recalibrate droplet size using the micrometer slide and reducing the duration of ejection by lowering the "period."
8. If too much time is lost and RNA is microinjected at the two-cell stage, the mosaicism that arises will be considerable (Fig. 3a).
9. Under the stereomicroscope the lanes will be clearly visible. Use a truncated microloader pipette tip to position embryos in the lanes. At this point it is prudent to eject a single RNA droplet and check it is still 1 nl on the micrometer slide. It is good to do this before every round of microinjecting as well in case the yolk from the embryos microinjected in the previous round has blocked the needle and causes <1 nl to be ejected in subsequent rounds of microinjecting.
10. Bright field is usually sufficient for identifying the cell and microinjecting but Nomarski optics can be used to improve contrast between the yolk and the transparent cell of the zebrafish embryo.
11. This movement is extremely fluid such that it is one move, once competent a rate of >50 embryos per minute is achievable. If the cell is missed and instead the RNA is deposited into the yolk of the embryo, then continue. During development most of the RNA from the yolk will be taken up into the cell. Especially when learning the technique the first few times it is easier to aim right under the cell, providing a wider target area, as the RNA will be taken up within minutes following injection through cytoplasmic streaming. If injecting DNA be aware that DNA is a considerably larger molecule and is therefore barely taken up through cytoplasmic streaming!
12. If development needs to be accelerated or decelerated for any reason then incubate at maximum 31 °C (faster development) or minimum 21.5 °C (slower development), respectively.

13. MS222 is sensitive to light and loses potency with time. For this reason, I often wrap the plastic dish with 0.1 % MS222 in E3 medium 0.01 % methylene blue in aluminum foil when intending to amputate many embryos.
14. Preferably cut on the lid so that the rim does not obstruct the motion of incision. The plastic will not break. A microscope with a small base is preferable as it provides free vertical movement of the arm when performing amputations.
15. In parallel I usually transfer an uncut control embryo to the plastic 10 cm dish with fresh 0.1 % MS222 in E3 medium with 0.01 % methylene blue mentioned in **step 6**.
16. The main focus area for improvement in this technique is accuracy to perform the amputation as close to the notochord as possible without damaging the notochord. The notochord does not regenerate, hence if nicked or amputated the embryo will die. Also, avoiding tearing of the fin tissue by pulling the surgical blade away from wound margin is desirable to simplify the analysis of regenerative outgrowth. The optimal incision motion is made using downward pressure to sever the caudal fin tissue and not slicing towards you, as this can create a pulling force on the fin tissue and lead to a sheared fin instead of a clean cut.
17. Preferably use a glass Pasteur pipette from this point forward as the amputated caudal fin readily sticks to plastic, causing damage to the caudal fin.
18. Use microloader pipette tips to position embryo with the posterior end flat on the bottom of the plastic dish. Nomarski optics can be used to improve contrast of the transparent caudal fin of the zebrafish embryo. For comparing different embryos it is paramount to use the same zoom settings on the stereomicroscope. Using the abovementioned Leica setup there is enough space to take a picture of both the amputated embryo and the uncut control in the same image.
19. Smaller than a 24-well plate is not recommended as the embryos have insufficient space to grow.
20. By performing the assay this way each uncut control is near enough at the same developmental stage as the amputated embryos and changes in embryonic growth rate can be accounted for.
21. The caudal fin of 5 dpf embryos is substantially bigger than at 2 dpf. Hence, each embryo for both amputated and uncut controls requires a separate image.
22. The chorion contains maternal DNA and proteins; this needs to be removed for accurate estimation of embryonic protein content.

23. The temperature is simply set to a comfortable temperature in which the embryo will survive. If using a 1.5 ml tube or multi-well plate it is easy to look under a standard stereomicroscope to check how much of the yolk has dissociated from the embryo.
24. If not deyolking, any excess system water or E3 medium can be removed from fin-clips or embryos respectively and directly subjected to snap-freezing in liquid nitrogen. By snap-freezing the tissue becomes more brittle, increasing homogenization efficiency. Can store tissue at this point at –80 °C.
25. Accompany a firm up-down motion with a gentle rotation for maximum efficiency. The tissue will shear. When removing the pestle some buffer or tissue may adhere to the head.
26. Creating air bubbles is practically unavoidable with this method but if the appropriate needle is used the tissue will be better sheared than it would have been with a mini-pestle. A small amount of suspension is always lost in the tip of the syringe which cannot be dispensed.
27. This removes quaternary and tertiary structures of proteins. Take into consideration that, after preparation with sample buffer and boiling, the sample may be very viscous, especially if little lysis buffer is used for many embryos. Upon such an occurrence the sample usually needs to be diluted with more sample buffer for accurate loading to be feasible. It is therefore very important to follow the guidelines set out in **step 9** to avoid this complication.

Acknowledgements

We would like to make a special mention of Miriam Stumpf who optimized sonication of zebrafish embryos in our lab and was kind enough to share her expertise. Also we thank Jelte Sikkens and Andreas van Impel for providing Fig. 3a and b respectively. We are grateful to all our colleagues, both within the den Hertog group and in the wider field of zebrafish research, for sharing their experiences with us and helping to make this chapter as comprehensive as possible with contributions from experts in zebrafish research.

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