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Method Article

Microinjection quality control in zebrafish model for genetic manipulations

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A B S T R A C T

Microinjection technique is one of the essential methodologies that are used widely in zebrafish research. Microinjection is utilized to perform genetic manipulations within the developing zebrafish model. Further, this technique is used to study a wide range of genetic diseases and gene of interest role in early developmental processes. Thus, quality control for microinjection is an essential factor to ensure experimental reproducibility and consistency. In this technical note, in vitro transcribed synthetic mRNA encoding green fluorescence protein (eGFP), and red fluorescent protein (m-cherry) as well as fluorescein and rhodamine fluorescent dyes were injected into a single-cell zebrafish embryo for volume quality control. Given the importance of having quality control system and methodology to yield similar genetic manipulation within the zebrafish embryo:

- We aimed to establish the unified delivery of injected material into zebrafish one cell stage embryo.
- We aimed to establish consistency of the injected volume into mineral oil droplets that will serve as a quality control parameter to conforms a quality control practice to ensure the reproducibility of the microinjection technique.
- The calibration of microinjection droplet size resulted in the visualization of fluorescent protein and dyes in the zebrafish embryo with precise volumes of delivered materials under the control of needle opening, injection pressure and time.

A R T I C L E I N F O
Method name: Quality control and reproducibility of microinjection material in zebrafish embryos achieving consistent genetic manipulation at one cell stage
Keywords: Zebrafish model, Microinjection, Droplet size, Genetic Manipulation
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Specifications table

Subject Area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Genetic manipulations within the developing zebrafish model
Method name:	Quality control and reproducibility of microinjection material in zebrafish embryos achieving consistent genetic manipulation at one cell stage
Name and reference of original method:	Rosen, J. N., Sweeney, M. F., Mably, J. D. Microinjection of Zebrafish Embryos to Analyze Gene Function. J. Vis. Exp. (25), e1115, doi:10.3791/1115 (2009).
Resource availability:	**Materials and reagents** • Phenol red (Sigma, cat#P0290, USA) • Petri dish • Phenyl Thio Urea (PTU) (P7629) • Egg water (E3): NaCl (Sigma, cat#S7653, USA), KCL (Sigma, cat#P9333, USA), MgSO4-7H2O (Sigma, cat#63138, USA), CaCl2-2H2o (Sigma, cat#C1016, USA) • Agarose (Sigma Aldrich, cat# A9539, USA) • Glass thin w/filament injection capillaries (World Precision Instrument, cat#TW100F-4, USA) • µTip Micropipette (World precision instruments cat#Tip05TW1F, USA) • Adhesive putty (tac patafix) • Mold (Eppendorf, Germany or Adaptive sciences tools, Cat # TU-1) • Micrometer - stage graticules S16 (VWR, cat#100499-308, USA) • Loading tips (Eppendorf, 20ul, cat#5242956.003, Germany) • Mineral oil (Sigma, cat#M5904, USA) • Blade • Parafilm • microscope slide • transfer pipette (VWR, cat#6124545) • DNA plasmid (Addgene, pCS2+8NmCherry, cat#34936, pSGEM-1 eGFP#44) • Subcloning efficiency chemi-competent cells: E.Coli (DH5α) (Thermofisher Scientific,18265017, USA) • Purelink Quick PCR purification kit (cat#K310002, Thermofisher Scientific, USA) • Mmessage Mmachine T7 transcription kit (Ambion, cat#Am1344, USA) • Mmessage Mmachine Sp6 transcription kit (Ambion, cat#Am1340, USA) • Restriction Enzymes: EcoRI (Thermofisher Scientific, cat#ER0271, USA), Nhel (cat#ER0971, Thermofisher Scientific, cat#ER0271, USA) • Rhodamine injection dye (Thermo Fisher Scientific, cat# D1816, USA) • Fluorescein injection dye (Thermo Fisher Scientific, cat# D1820, USA). **Equipment's** • Hood (Thermo scientific,1300 SERIES A2, USA) • Needle puller (Narishige, PC-10, Japan) • Picoliter injector (Harvard apparatus, Warner instrument, PLI-100A, USA) • Air source • Microscope (ZEISS, Stemi 2000-C, Lumar V.12, Germany) • Camera (ZEISS, Axiocam ERc 5s, USA) • ZEN blue Software (lite2.5, Germany)

Method details

Ethical statement

All protocols used in this report were approved by the local Animal Care and Use Committee and conform to the Zebrafish Policy published by the Qatar Ministry of Public Health that follows the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. (http://www.qu.edu.qa/static_file/qu/research/documents/qu_iacuc/MOPH%20Policy%20on%20Zebrafish.pdf). Experiments performed on zebrafish were approved by the IACUC Office of Qatar University (QU-IACUC, 26-2/2018-REN1) and Qatar Foundation (QF-IACUC, EVMC-2020-006) under MOPH guidelines (IACUC-SIDRA-2020-0006).

Zebrafish housing, husbandry, and care

Zebrafish (*Danio rerio*) wildtype (AB strain) were obtained from European Zebrafish Resource Center (EZRC, Germany). The optimal condition of housing density of 3-5 fish per liter in a recirculating aquaculture system under standard environmental conditions of temperature at 27 °C, conductivity at 800 µs and pH at 7.5 with 14 h light starting at 7:00 am and 10 h dark cycle. To maintain the optimal environmental conditions, water exchanges of 10% per day was performed and water quality checks in a weekly basis. The health of the zebrafish was monitored on a daily basis and if any signs of sickness, preventive measures as per the housing guide were followed.

Study design and sample size

For zebrafish injection experiments, embryos were collected in N-phenylthiourea (PTU) (Sigma, P7629) or egg water (E3) media. A total number of 100 embryo were used per each condition per experiment and done in a triplicate. Injected embryos were raised at 28 °C incubator. Larvae at 24, 48, and 72 h post-fertilization (hpf) were assessed for uniformed injection by intensity examination and imaging accordingly to visualize the fluorescent protein. The selection of representative images was randomized. At end point of experimentation, zebrafish larvae were euthanized by administreing of Tricaine MS-222 (Western Chemical, 1029D11) anesthetic agent (200 mg/l) followed by chilling on ice. Upon euthanasia, zebrafish carcasses were disposed of as pathological medical waste.

Part 1: DNA preparation

- mCherry pCS2+8N plasmid was transformed using DH5α competent cells and then purified following a previously published protocol [10].
- mCherry pCS2+8N (Figure S1) and eGFP pSGEM-1 (Fig. S2) plasmids were linearized at the 3' end EcoRI and Nhel restriction enzymes and transcribed under the SP6 (cat#Am1340) and T7 (cat# AM1344) promoter, respectively following kits protocol.

Part 2: Embryo collection

- Wildtype AB zebrafish were set up in breeding tanks by separating males and females using a plastic divider, the night before injection.
- Dividers were pulled from some tanks (to make sure the eggs will not pass the single-cell stage) the following morning, after turning the room lights on, and fish were allowed to mate without disruption during mating time.
- Eggs were collected using a strainer and rinsed with phenylthiourea or egg water (E3) media. Then they were poured into a Petri dish with PTU or egg water (E3) media to line up the fertilized eggs into the ramps of the pre-casted injection plate using a transfer pipette.

Part 3: Microinjection setup

- Microinjection plate (2% mold) was prepared using 0.6 g agarose and 30ml PTU or Egg water (E3) media, then heated in a microwave and poured into a Petri dish; after that, ramps were created by laying the mold over the agarose.
- To ensure consistency and reproducibility, the important factor is to use the same brand needle with the same diameter and the same length to be able to eject the same volume per experiment. In this protocol, we selected capillaries, 4 in. with outer diameter of 1.0 mm with filament (TW100F-4, World precision instruments, USA) or pre-pulled needles with an internal tip diameter of 0.5 µm (TIP05TW1F, World precision instruments, USA).
- Before injection, a glass capillary with a 1.0 mm O.D. was pulled (Pulling temperature 69.9 °C, pulling force: two pieces of light weights and two pieces of heavy weights provided by manufacturer Narishige International, USA) into two equal needles using a micropipette puller (Narishige, PC-10) and stored in a 100 mm Petri dish by laying over adhesive putty (Fig. 1A).

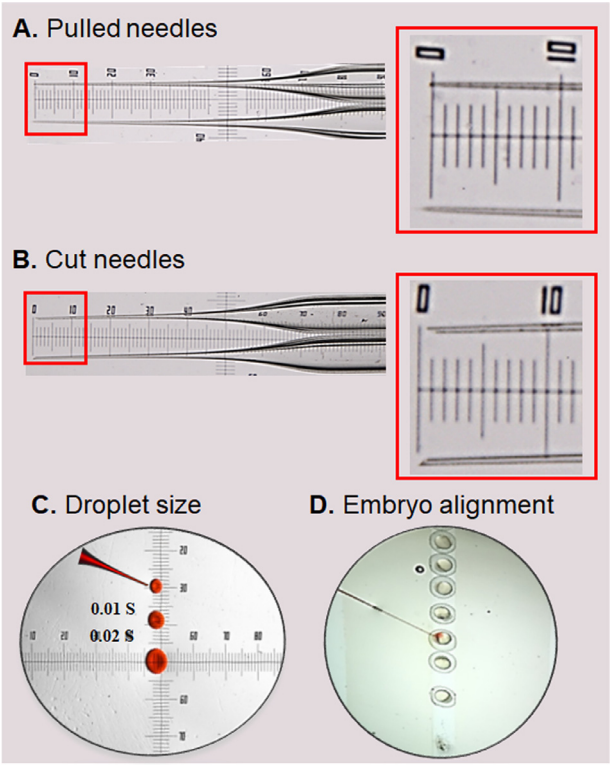

Fig. 1. Microinjection setup for needle and droplet calibration**. A**. The length of the pulled needle before cutting. **B**. Needle selection: cutting the tip of a pre-pulled needle with a sharp blade on a scale micrometer slide wrapped by parafilm under the microscope to produce a slant needle with similar opening. **C**. Injection with a slant needle backfilled with phenol red to produce a droplet. Volume determination by injecting into mineral oil placed on a micrometer. **D**. Fertilized embryos aligned into the ramps of pre-casted injection plate.

- To prepare the tip of the needles, a microscope scale micrometer slide was wrapped with parafilm. Then the tip of the needle was cut with a sharp blade under the microscope in a slant degree. The cut needles were selected to have a consistent and reproducible fine tip through visualization on the scale micrometer slide (Fig. 1B).
- The pre-cut slant needle is fine enough to pierce the chorion and yolk and to expel a consistent and reproducible bead size ideally less than10% of the embryo volume which is $\leq$4.2 nl [11,12].
  For best RNA distribution, usually, 2 µl is used for 1-cell and then the volume is decreased for later stages (4, 8, 16 cells, etc.) accordingly to avoid their damage when performing single blastomere injections as those cells are smaller [13]. By ensuring needle tip quality, the injection process will be easier, and the result will be consistent.
- The air source and microinjector were turned on. Needle was backfilled with phenol red which helps to visualize the injection procedure and inserted tightly within the housing of the microinjector holder of the Pico-Injector Microinjection System (Harvard apparatus, Warner instrument, PLI-100A) that forces the injection solution using foot pedal and air pressure. Then, injection time and pressure were adjusted to maintain a consistent bead size and to prevent the medium from flowing back into the needle and diluting or contaminating the injection solution.
- To calculate injection volume, a drop of mineral oil was placed on a micrometer. Then phenol red was injected into the oil using the foot pedal, and the bead size was monitored and measured (Fig. 1C).

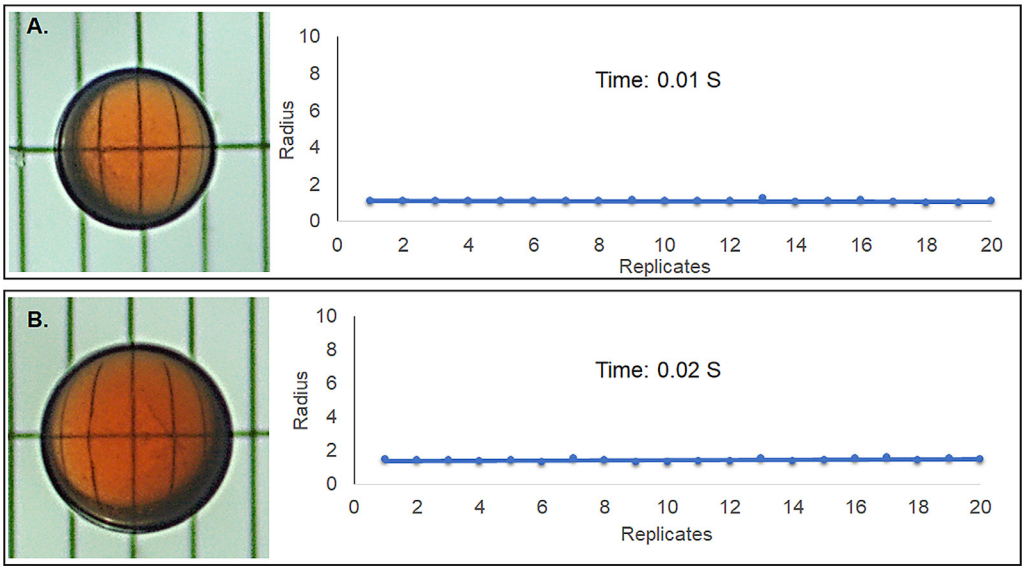

Fig. 2. Measurement of ejected droplet size. **A**. Graph representing 0.01 injection time: radius average = 1.12-in. *unit measurement= 0.33-in., radius average/unit= 3.41µm, standard deviation (SD) ± 0.048. **B**. Graph representing 0.02 injection time: radius average = 1.45-in., *unit measurement = 0.33-in, radius average/unit = 4.39 µm, SD ± 0.072. *Distance between two lines using stage micrometer and oil for droplet size measurement.

- The following formula was used for calculation: Volume in nanoliters = .17952(*) x (internal tip diameter (I.D.) in micron)3 x (Pressure in Pounds per Square Inch (psi)) x (Time in seconds) (Fig. 2A, B).
- The aligned embryos were injected using an injection plate and the pre-cut slant needle (Fig. 1D).

Part 4: Fluorescent protein RNA injection

- The needle was backfilled slowly toward the needle tip with 2 µL of the transcribed m-RNA and phenol red using a microloader pipette (cat #5242956.003).
- The injection material was injected into the yolk of zebrafish embryos using ~ 1.5 ug/ul mRNA at their one-cell stage, with a consistent injection time of 0.01 second (s) and injection pressure of 14.7 Pound per square inch (psi).
- The volume in nanoliters was calculated as the following = $0.17952(1)^3$ x (14.7) x (0.01) =0.026 nl.
- At the same time, two dilutions 1:10 and 1:20 of fluorescent dye were injected into zebrafish embryo using rhodamine injection dye MW10,000 (Thermo Fisher Scientific, USA cat# D1816) and fluorescein injection dye MW10,000 (Thermo Fisher Scientific, USA, cat# D1820).
- To prepare fluorescent injection solutions of rhodamine and fluorescein injection dyes. We have resuspended rhodamine and fluorescein in nuclease-free water to make a stock concentration. For microinjection, the stock was diluted in nuclease-free water. Note: fluorescent dye begins to express in zebrafish embryo immediately from 1 cell stage post-fertilization. The injected embryos were incubated at 28.5 °C incubator for further development. Once inside the cells, the injected mRNA will be distributed throughout the developing embryos and will translate, yielding a fluorescent green or fluorescent red protein.

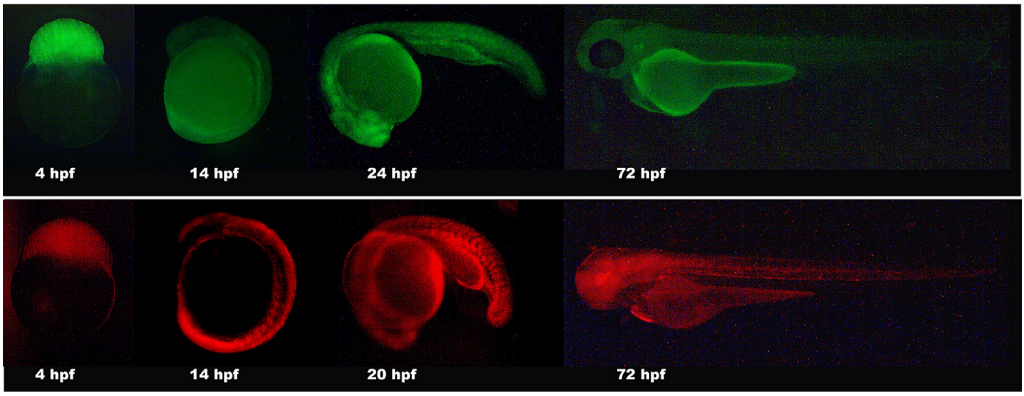

Fig. 3. Representative images of injected embryos with synthetic e-GFP and m-Cherry fluorescent RNA. Injected embryos screened for GFP fluorescent protein and m-cherry fluorescent protein at different stages through development staged by hours post-fertilization (hpf). Images captured using LumarV.12 stereomicroscope and Axiocam camera at 20x objective.

Part 5: Embryo screening

- Lumar V.12 stereomicroscope was used to screen fluorescent embryos using two channels (excitation 650 nm and emission 669 nm for m-cherry and 488nm and 509nm for e-GFP, respectively) at a different developmental interval time (Figs. 3 and 4).

Part 6: fluorescence signal analysis

- Imaging is performed using a unified fluorescence exposure intensity.
- The exposure time is fixed per imaging set.
- To analyze fluorescence signal, a specific region of the embryo is selected by drawing a rectangle. Then the same rectangle area is used to measure the fluorescence intensity across all embryos at the same selected region.
- The imaging software is used to calculate the mean intensity value for the selected region (Fig. 5).

Statistical analysis

Statistical analysis was performed by calculating the mean and standard deviation (SD) using GraphPad Prism software, version 8 prism.

Results

The unified volume, pressure and diameter of the injection needle lead to the reproducibility of the droplet size per injected material into the mineral oil droplet. As a result, our controlled methodology resulted in the unified fluorescent intensity of the injected green and red fluorescent proteins.

In vitro *transcribed mRNA of fluorescent proteins*

To assist in performing this procedure, we used plasmids encoding fluorescent proteins. The linearization was successful, and the *in vitro* transcription kit was used to produce both the green fluorescent protein (eGFP) and m-Cherry as controls.

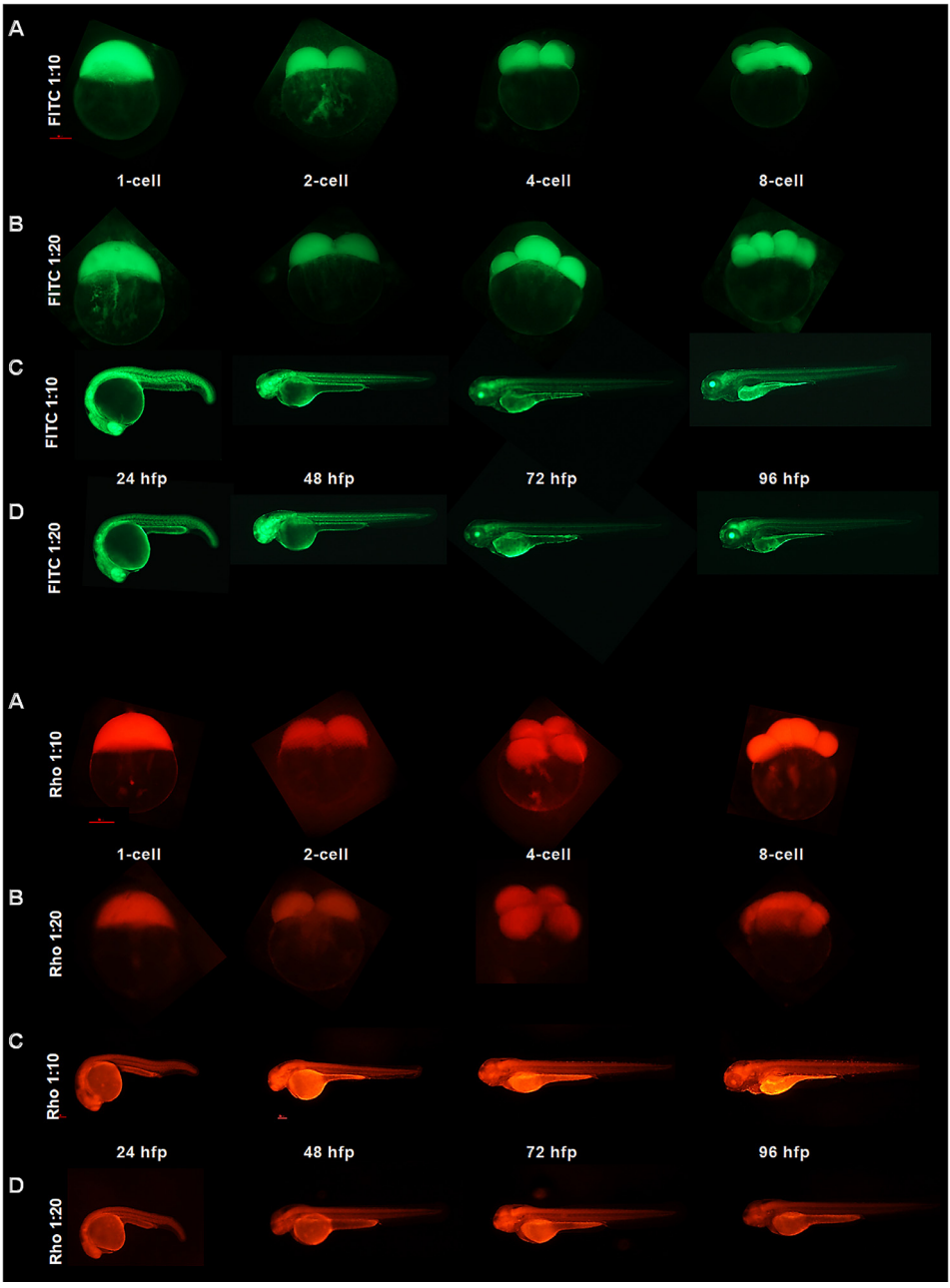

Fig. 4. Representative images of injected embryos with fluorescein and rhodamine fluorescent dyes. The injected embryos were screened for the fluorescent dyes' (fluorescein and rhodamine) intensity at early stages of development: **A.** Dilution factor was 1:10 from original stock. **B.** Dilution factor was 1:20 from original stock. Representative images were captured at 80x objective using Lumar V.12 stereomicroscope.
Fluorescent dye intensity at different developmental stages was presented by age at 24, 48, 72, and 96 h post fertilization (hpf). **C.** Fluorescent dyes dilution 1:10. **D.** Fluorescein and rhodamine fluorescent dyes dilution 1:20. Images were captured using Lumar V.12 stereomicroscope and Nikon camera at 40x objective.

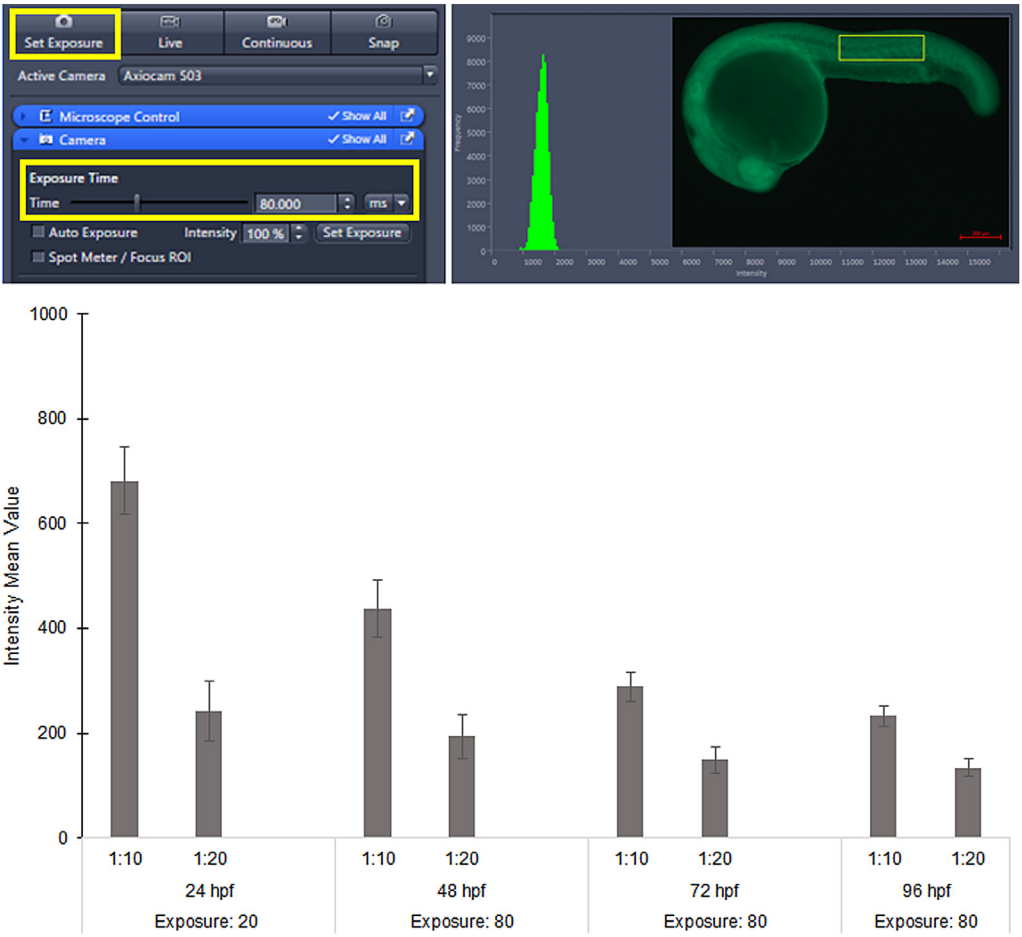

Fig. 5. Fluorescence signal analysis. Exposure time set for a unified exposure intensity using imaging software. A specific area selection for quantifying the fluorescence signal intensity. Exposure intensity and time setting with fluorescent intensity histogram of FITC injected zebrafish embryo at 24hpf. Column graph showing mean fluorescent intensity quantification of representative fluorescein fluorescent dye at two dilutions, 1:10 and 1:20, across zebrafish development. Number of embryos analyzed were n= 8–10 per age group.

Microinjection setup preparation

We successfully obtained a uniformed cutting for the injection needles under the microscope and utilizing the micrometer (Fig. 1). It is important to get a unified cut for the slanted needle to have an equal diameter of the needle tips.

The controlled conditions for the microinjection setup which include the needle tip, injection time and injection pressure resulted in a uniformed and consistent droplet size based on the injection time (Fig. 2). Our analysis showed a mean radius length of 1.126 (3.84 µm) for droplet diameter of 0.01 second (s) injection time with standard deviation (SD) ± 0.048. Increasing the injection time to 0.02 s resulted in a droplet diameter mean radius length of 1.45 (4.94 µm) with SD ± 0.072.

The injection of single-cell embryo injection was performed under the stereomicroscope and visualized by phenol red ejections. Screening of injected zebrafish embryos resulted in the expression of fluorescent proteins. The injected m-cherry RNA and eGFP RNA were translated to fluorescent proteins (Fig. 3). The injected embryos displayed fluorescence and were imaged at the different developmental stages confirming our controlled and established procedure. It is established that GFP fluorescent protein can be detected at 6 hours in the injected embryos at the shield stage.

Fluorescent dye

The injection of fluorescent dyes into one-cell stage zebrafish embryos facilitated the observation of fluorescent intensity over time to achieve the reproducibility and control of the microinjection procedure (Fig. 4). This method can be used to confirm the accuracy of the injection and the proper delivery of the injected reagents. The injected zebrafish groups displayed fluorescence and were imaged at the different developmental stages using the same exposure reflecting the intensity of the fluorescent dye dilution confirming our controlled and established procedure (Fig. S3). Additionally, our protocol showed a consistency of the mean fluorescent intensity. The quantification of fluorescein fluorescent dye across zebrafish development showed consistent fluorescent intensity measured at two dilutions, 1:10 and 1:20: 680, 240 (24 hpf) 440, 195 (48 hpf) 290, 150 (72 hpf) and 230, 130 (96 hpf) respectively.

Discussion

Modelling human disease in the zebrafish model provides a convenient and powerful platform to study human genetic diseases and disorders. Microinjection is widely used to achieve genetic manipulations in the one-cell stage zebrafish embryo at the earliest stages of development.

The functional characterization of genotype-phenotype associations of the human genetic diseases have contributed to linking genomic sequences or function of the gene with complex clinical presentation of patients [1–4]. The unique power of zebrafish model has contributed to unravelling novel connections between genetics and disease and allowed real time tracking of the genetic contribution of an individual gene or multiple genes in relation to the human disease etiology [5,6]. The genetic manipulation of zebrafish model leverage the visualization of resulting traits by the examination of the model morphology, physiology and behavior through well-established assays [7–9].

Microinjection consistency is the crucial factor to be considered for reproducing the genetic manipulation by delivering the same volume into the one-cell stage embryo. In this protocol, the established consistency of the injected volume into mineral oil droplets served as a quality control parameter to ensure the reproducibility of injecting the in *vitro* transcribed mRNA. Our calibrated microinjection process resulted in a consistent intensity of the green eGFP and the red m-Cherry fluorescent proteins in injected zebrafish embryos as well as the selected fluorescent dyes.

Therefore, the quality control of microinjection should be considered before each injection by implementing a quality control system for precise injections to achieve a uniformed expression of the injected material. The quality control of the microinjection technique will be of particular use in genetic manipulations where volume is challenging and critical to achieve reproducibility and unified delivery, modification and expression in the zebrafish manipulated embryo.

Conclusion

In this report we established calibration, consistency, and reproducibility of injected material into the zebrafish single cell embryo. This is very crucial for the uniformity of genetic manipulations. The injected material volume delivered to the zebrafish embryo is affected by different factors: the tip of the needle, time, and pressure. The microinjection was performed at one cell stage using synthetic RNA for the delivery of green fluorescent protein and m-cherry fluorescent protein. Subsequently, these embryos successfully expressed the fluorescent protein up to 72 hpf serving as a quality control of microinjection reproducibility for the delivery of the exact volume.

Declaration of Competing Interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

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Authors' contributions

Ms. Abdelrahman carried out the RNA transcription, microinjection, imaging, graphical abstract drawing and drafted the manuscript. Mr. Hasan participated in the imaging. Dr. Da'as supervised the project, designed the study, and reviewed the results and manuscript. All authors read and approved the final version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101418.

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