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Maximizing single cell dissociation protocol for individual zebrafish embryo

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ABSTRACT

Single-cell sequencing has revolutionized our understanding of cellular heterogeneity and cell state, enabling investigations across diverse fields such as developmental biology, immunology, and cancer biology. However, obtaining a high-quality single-cell suspension is still challenging, particularly when starting with limited materials like Zebrafish embryos, a powerful animal model for studying developmental processes and human diseases. Here, we present an optimized protocol for isolating single cells from individual zebrafish embryos, offering a valuable resource for researchers interested in working with limited starting material. The protocol facilitates unique investigations utilizing individual embryos, such as inter-individual genetic differences and embryo-specific lineage tracing analysis. Using a refined single-cell isolation protocol alongside zebrafish as a model organism, researchers can access a resource for exploring the emergence of all types and states of cells, advancing our understanding of cellular processes and disease mechanisms.

Specifications table

Subject area:	Biochemistry, genetics and molecular biology
More specific subject area:	Developmental Biology
Name of your protocol:	Single Cell Dissociation protocol for individual Zebrafish embryo
Reagents/tools:	• Egg water (home-made) • Methylene blue (Sigma Aldrich-03,978–250ML) • Pronase (1 mg/ml) (Sigma Aldrich-10,165,921,001) • 10X PBS (free of calcium chloride and magnesium chloride) (Gibco-70,011–044) • 1X DPBS (free of calcium chloride and magnesium chloride) (Gibco-14,190–144) • Nuclease-free water (Invitrogen-10,977–023) • 1X DMEM (Gibco-11,965–092) • BSA (Sigma-A9576) • Collagenase (100 mg/ml) (Millipore sigma-SCR103) • 0.25 % trypsin + EDTA(1x) (Thermo fisher-25,200,056) • FACSmax solution (Gentalis-T200100) • Optiprep (Sigma Aldrich-D1556–250ML) • Trypan blue (Invitrogen-T10282)
Experimental design:	An optimized, chemical and mechanical based protocol for isolating single cells from individual zebrafish embryos.
Trial registration:	Non applicable
Ethics:	The zebrafish care and experimental procedures complied with the protocols approved by the institutional animal care and use committee at the University of California San Francisco (UCSF).
Value of the Protocol:	The protocol facilitates unique investigations utilizing individual embryos.

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Background

Zebrafish embryos are an excellent model organism for biomedical research [1–3]. Combining zebrafish with state-of-the-art single-cell technologies opened many possibilities, including studying gene expression patterns during development [4–7], deciphering cellular lineages [8–11], unveiling cellular heterogeneity [12–14], and exploring the interplay between different cell types [15–17]. While numerous protocols exist for generating single-cell suspensions, they require pooling multiple embryos to obtain sufficient starting material for downstream analysis [18]. While this pooling strategy serves its purpose in certain studies, it sacrifices the ability to achieve single-embryo resolution, which is essential for a wide range of investigations, including inter-individual comparisons [11], lineage tracing, clonal analysis, drug discovery, and precision medicine studies. Furthermore, pooling embryos can introduce animal-animal variability and compromise the data quality, especially if a few embryos behave as outliers. This limitation led us to develop a new protocol to increase dissociated cell yield from a single zebrafish embryo, optimizing the process across developmental stages from 10 hours post-fertilization (hpf) to 10 days post-fertilization (dpf), tailored to your specific stage of interest. This is done using gentler dissociation methods for younger stages and more aggressive approaches for older embryos. The protocol was optimized for zebrafish embryos but can be adapted for other challenging organisms with limited starting material.

This paper presents our solution for obtaining a high-quality single-cell suspension from a single zebrafish embryo, as highlighted in (Fig. 1a and b). We utilized single-cell sequencing to showcase our protocol's effectiveness, as evidenced by our recently released Zebrahub dataset [11] (data explorable at https://zebrahub.ds.czbiohub.org/transcriptomics). Furthermore, by leveraging techniques such as proteomics, FACS sorting, and drug screening, our protocol enables a broader range of studies, with the ability to achieve single-embryo resolution and precise temporal comparison (Fig. 1c). All together, we believe our protocol will significantly benefit researchers where obtaining adequate single cell suspensions poses a significant hurdle.

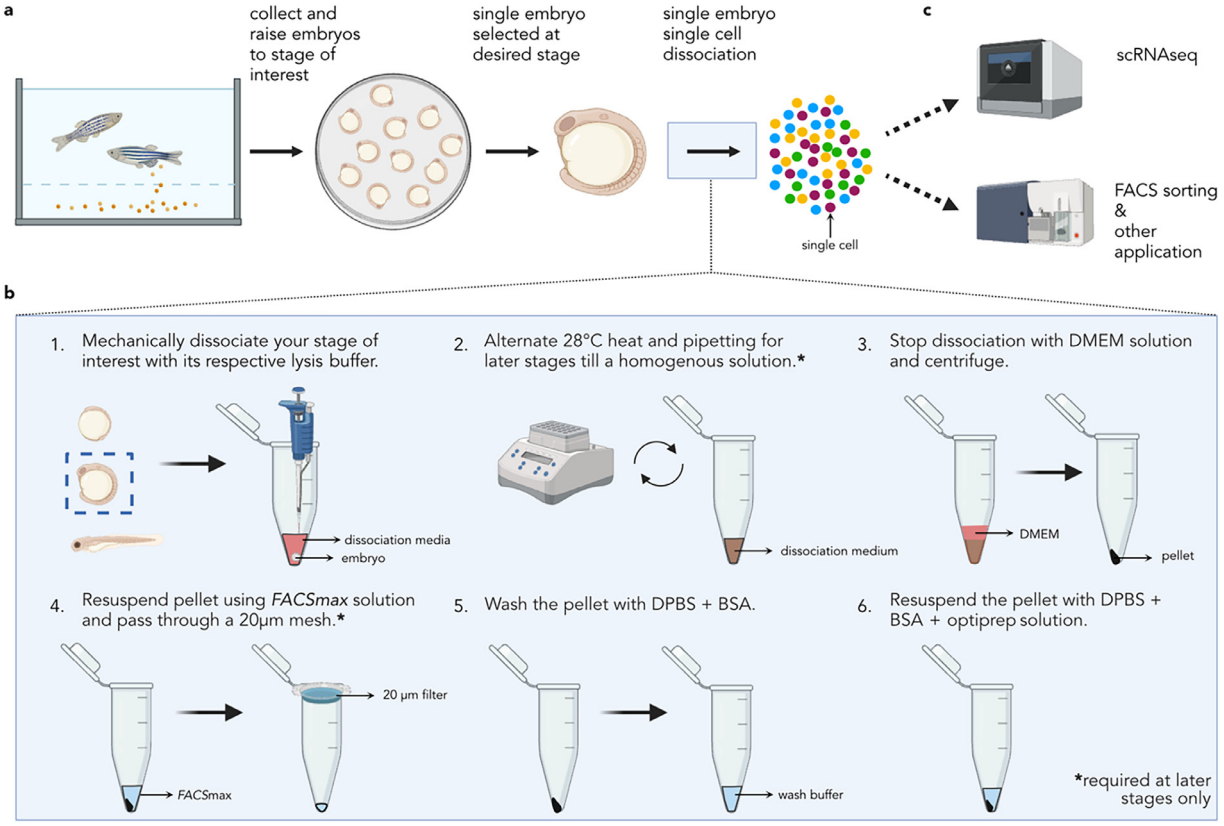

Fig. 1. Summary of the protocol to obtain single cells from individual embryos. (a) Depicts the step-by-step procedure of isolating individual cells from embryos, encompassing fish mating, embryo phenotyping, collection at the desired stage, and obtaining single cells through dissociation. (b) Close-up visual representation of the dissociation pipeline employed to extract single cells from individual embryos. (c) Explores the diverse range of potential applications for utilizing these single cells in various research studies.

Description of protocol

Reagents

- Egg water (home-made)
- Methylene blue (Sigma Aldrich-03,978–250ML)
- Pronase (1 mg/ml) (Sigma Aldrich-10,165,921,001)
- 10X PBS (free of calcium chloride and magnesium chloride) (Gibco-70,011–044)
- 1X DPBS (free of calcium chloride and magnesium chloride) (Gibco-14,190–144)
- Nuclease-free water (Invitrogen-10,977–023)
- 1X DMEM (Gibco-11,965–092)
- BSA (Sigma-A9576)
- Collagenase (100 mg/ml) (Millipore sigma-SCR103)
- 0.25 % trypsin + EDTA(1x) (Thermo fisher-25,200,056)
- FACSmax solution (Gentalis-T200100)
- Optiprep (Sigma Aldrich-D1556–250ML)
- Trypan blue (Invitrogen-T10282)

Equipment

- Net
- Petri dishes (GenClone-25–202)
- Glass petri dish (Fisher scientific: S82820)
- Lo-bind Eppendorf tubes (Eppendorf-022,431,021)
- Pipettes (Rainin-2,20,200,1000)
- Sterilized filter Pipette tips (Rainin-2,20,200,1000)
- Glass Pasteur pipette (Fisher scientific: 22–037–514)
- 20 μm eppendorf fitted mesh (PluriSelect: SKU: 43–10,020–40)
- Heat shaker (Eppendorf: ThermoMixer C EP5382000023)
- Tabletop centrifuge (Eppendorf: Centrifuge 5424 R)

Reagent setup

- **1x PBS**

  Dilute 10x PBS with nuclease-free water and store at room temperature.

- **Egg water**

  1.5 ml stock salts added to 1 L distilled water = 60 μg/ml final concentration.
  Stock salts: 280 g Instant Ocean Sea Salts (Aquarium Systems, Inc.) dissolved in 2 liters of distilled water

- **Pronase solution**

  10 mg pronase is added to 10 ml egg water and vortexed till completely dissolved.

- **Pre-coat buffer: 1x DPBS + 2 % BSA**

A 30 % BSA stock solution is used to prepare the dilution. BSA powder can be used as an alternative to make the working solution. The solution is placed on ice post-preparation and stored at 4 ˚C for a maximum of 2 weeks.

- **Dissociation media (10 - 24 hpf): FACSmax solution**

The solution should be allowed to thaw completely before use. To avoid multiple freeze-thaw cycles, it is recommended to divide the solution into smaller 2 ml aliquots.

- **Dissociation media (2–10 dpf): 0.25 % Trypsin + 100 mg/ml collagenase**

Stock solution: 20 μl aliquots of 100 mg/ml collagenase solution are prepared and stored at −20 ˚C.
Working solution: Depending on the relevant stage of interest, fresh quantities of trypsin and collagenase solutions are prepared and kept on ice.

- **Stop solution: DMEM + 1 % BSA**

30 % BSA stock solution is used to prepare the dilution. BSA powder can be used as an alternative to prepare the working solution. The solution is prepared fresh every time.

- **Wash buffer: 1x DPBS + 1 % BSA**

30 % BSA stock solution is used to prepare the dilution. BSA powder can be used as an alternative to prepare the working solution. The solution is placed on ice post-preparation and can be stored at 4 ˚C for up to 2 weeks.

- **Resuspension buffer: 1x DPBS + 0.5 % BSA + 18 % optiprep**

30 % BSA stock solution is used to prepare the dilution. BSA powder can be used as an alternative to prepare the working solution. To ease the handling of the viscous Optiprep solution, it is recommended to slightly trim the end of the pipette tip for improved pipetting. The solution is placed on ice post-preparation and can be stored at 4 ˚C for 2 weeks.

Equipment setup

- Cool down the centrifuge to 4 ˚C.
- To minimize cell retention and reduce the risk of cell loss, pass 500 µl DPBS through an Eppendorf-fitted 20 µm mesh.

Procedure

Embryo crossing

Plan your crossing in advance depending on what stage you want the embryos for the experiment. Cross a pair of fish and allow them to lay embryos. The embryos are collected and grown at 28 ˚C until they reach the desired stage of interest. To collect embryos at a specific stage of interest, the mating can be controlled temporally with the help of a divider.

Before you start

- Pre-cool centrifuge to 4 ˚C.
- Precoat 4 Eppendorf tubes with DPBS + 2 % BSA. This is done by adding 800 µl per Eppendorf tube and allowing it to sit for 15 minutes (min) at room temperature (RT). The solution is then removed.
- Set up a heat block to 28 ˚C.
- Heat up DMEM + 1 % BSA on the heat block (28 ˚C).

Cell dissociation for embryos between 10 hpf and 24 hpf

DURATION: 30 min

1. Dechorionate the embryos in the small imaging petri dish (100 × 20 mm) with 10 ml 1 mg/ml of Pronase. Allow the embryos to stay in the pronase solution for 2 min or until you see the chorions soften. The petridish can be tilted slightly to ensure all the embryos are fully submerged in Pronase. Pinching the chorions with forceps and checking if they wrinkle is a good indication that they are ready for washing.
2. Remove the pronase solution post-incubation and add 20 ml egg water in the petri dish to wash the embryos. *Tips: Tilting the dish can help with removing the media.*
3. Change the egg water to wash the embryos once more.
4. Transfer the embryos to the small petri dish (75 × 15 mm) and gently aspirate the embryos up and down using a glass Pasteur pipette until the chorion is released.

Caution: Younger embryos must be handled carefully as they can be fragile. Harsh pipetting can lead to embryos getting deformed or dying.

Tips: Use a glass petri dish to dechorionate embryos. This helps with the embryo's survival.

Tips: If the chorions are not released, centrifuging them for 30 s at 300 Relative centrifugal force (rcf) can help.

5. Carefully transfer one embryo at a time into separate Eppendorf tubes and add 50 µl of FACSmax solution per tube (Fig. 2a).
6. Flick and pipette the tube with a 200 µl pipette tip 10–15 times or until the suspension is entirely homogenous (Fig. 2b).

Optional: For 24 hpf - the sample can be alternated between 30 s of pipetting and 30 s of heat block at 28 ˚C if not fully dissociated.

7. Centrifuge samples with the following conditions:
   - Bud stage to 12 hpf (5 somites), we applied 300 rcf for 2 min at 4 ˚C (Fig. 2c).
   - 14 - 24 hpf (10 - 30 somites), we applied 500 rcf for 5 min at 4 ˚C (Fig. 2c).
8. Carefully remove the supernatant and resuspend the pellet with 100 µl DPBS + 1 % BSA.

Caution: Always remove the solution from the side opposite to the pellet. This prevents the pellet from becoming loose and helps reduce the number of cells lost.

9. Centrifuge the tubes for:
   - Early stages up to 12 hpf, we applied 300 rcf for 2 min at 4 ˚C (Fig. 2c).
   - 14 - 24 hpf, we applied 500 rcf for 5 min at 4 ˚C (Fig. 2c).
10. Carefully remove the supernatant and resuspend the pellet in 25 µl DPBS + 0.5 % BSA + 18 % optiprep.

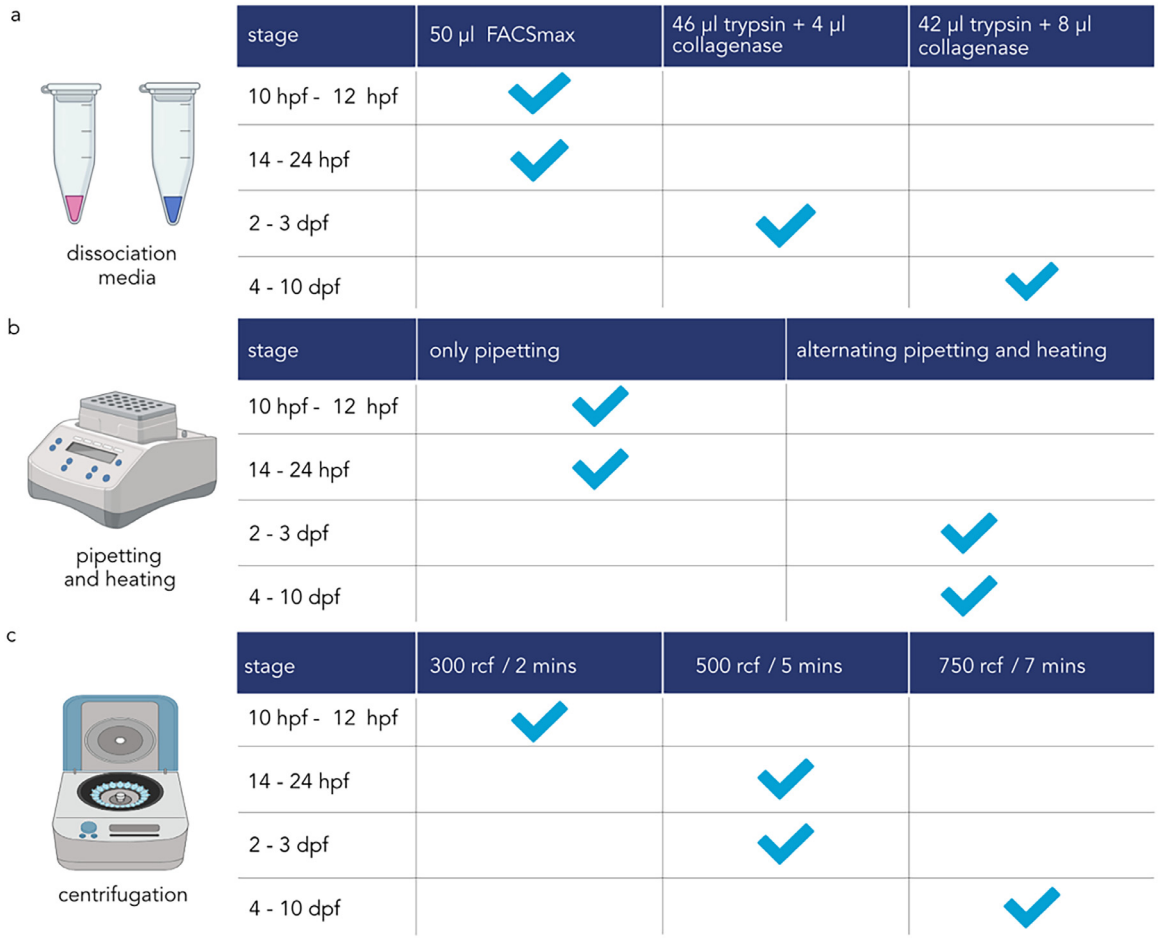

Fig. 2. Overview of the different conditions to obtain single cells based on the stage of interest. (a) Dissociation media preparation used at different developmental stages. (b) Dissociation technique used at each stage. For the earlier time points, dissociation can be adequately achieved through mechanical dissociation via pipetting, while late points require a combination of pipetting and heat incubation. (c) Required centrifugation speed and time for the time points across development.

Dissociation for embryos between 2 dpf and 10 dpf

DURATION: 45 min

1. To ensure embryos are unconscious and immobile, place the Petri dish on ice for 10–15 min. We avoid using tricaine or bleach for euthanasia prior to this step to prevent any interference with subsequent downstream data due to chemical residues.
2. In one Eppendorf tube:

   - For 2–3 dpf embryos, add 46 µl of trypsin + EDTA and 4 µl of 100 mg/ml collagenase (Fig. 2a).
   - For 4–10 dpf embryos, add 42 µl of trypsin + EDTA and 8 µl of 100 mg/ml collagenase (Fig. 2a).

3. Mechanically pipette the embryos using a 200 µl tip 10–15 times or until at least 80 % dissociated approximately. Only harder tissues (eyes, notochord) are visible at this point. Place the tube on the heat block for approximately 1 min. Alternate between the pipetting and heating 2–3 times until the suspension is entirely homogenous with no tissue remnants (Fig. 2b). (Additional repetitions with harsher pipetting may be needed for 5–10 dpf). Add 50 µl DMEM + 1 % BSA and leave the tubes on the heat block till all the replicates are dissociated.

Caution: Ensure the heat block is at 28 ˚C and shaking at 300 rpm. This helps dissociate the tough tissues easily — especially the eyes and spinal cord, which take longer to dissociate completely.

Tips: Position the Eppendorf tube in the direction of the light source and employ pipetting to verify the absence of any remaining tissue fragments. If no visible remnants are detected, the embryo has been uniformly dissociated.

4. Centrifuge the tubes for:

Table 1
Minimum cell count and viability required for each stage.

Stage of interest	Minimum cell count / µl	Minimum Viability %
10 hpf	250	75 %
12 hpf	500	75 %
14 hpf	500	75 %
16 hpf	500	75 %
19 hpf	600	75 %
24 hpf	1000	75 %
2 dpf	1000 - 2000	70 %
3 dpf	1000 - 2000	70 %
5 dpf	1000 - 2000	70 %
10 dpf	1000 - 2000	70 %

- For 2–3 dpf, we applied 500 rcf for 5 min at 4 ˚C (Fig. 2c).
- For 4–10 dpf, we applied 750 rcf for 7 min at 4 ˚C (Fig. 2c).

Caution: If a pellet is not seen, centrifuge for 2 additional min at 750 rcf.

5. Very carefully remove the supernatant, then resuspend the pellet in 100 µl of FACSmax solution.

Caution: Keep the replicates on ice while working on the other samples. Keeping samples at room temperature for extended periods may degrade the RNA.

6. Add the suspension to a pre-washed 20 µm filter mesh (see equipment setup) and conduct a brief 1 min centrifugation at 500 rcf. Then, remove the filter from the Eppendorf tubes.
7. Centrifuge the tubes for:

- For 2–3 dpf, we applied 500 rcf for 5 min at 4 ˚C (Fig. 2c).
- For 4–10 dpf, we applied 750 rcf for 7 min at 4 ˚C (Fig. 2c).

8. Carefully remove the supernatant, then resuspend the pellet in 100 µl DPBS +1 % BSA.
9. Centrifuge the tubes for:

- For 2–3 dpf, we applied 500 rcf for 5 min at 4 ˚C (Fig. 2c).
- For 4–10 dpf, we applied 750 rcf for 7 min at 4 ˚C (Fig. 2c).

10. Carefully remove the supernatant, then resuspend the pellet in the desired amount of DPBS + 0.5 % BSA + 18 % optiprep solution. For scRNAseq we recommend 25 µl.

Counting of cells
TIMING: 15 min

1. Count cells using a cell counter. It is advantageous if it is an automatic counter capable of generating live/dead cell counts. We add 10 µl of Nuclease-free water + 5 µl of Trypan blue to 5 µl of dissociated sample for the counting dilution.

Caution: Ensure the sample is pipetted gently before counting. This is essential as cells tend to settle at the bottom of the tube.

2. Dissociated cells can be used for subsequent downstream processing. High-quality cell preparation should exceed the minimum requirements indicated in Table 1.

We next compared cell yields of our optimized protocol for individual embryos with previously published protocols using pooled embryos which we adapted for a single embryo (Fig. 3a) [10,18,19]. In our hands these protocols were very inefficient for single embryo dissociations, and did not achieve the minimum cells needed for downstream applications (Fig. 3b).

Troubleshooting

Step	Problem	Possible reason	Solution
Dechorionation: 10 hpf – 24 hpf	Embryos die during the process.	Left in pronase solution for too long. Embryos not washed properly post pronase incubation. Harsh washing of embryos.	Do not over incubate embryos in pronase solution as it can be toxic for the embryos. 2 min is ideal for young embryos. Wash out the embryos with egg water at least 2 times to remove the residual pronase solution. Young embryos are very fragile post dechorionation, they should be handled very gently.
10 hpf – 16 hpf	Not being able to see a pellet post centrifugation	Started off with low number of cells and during the dissociation process possible cell loss is expected	*Re*-centrifuge the sample for a minute more at 400 rcf to settle all the cells at the bottom. If even a small pellet is still not seen, leave 10 µl of solution at the bottom of the eppendorf while discarding the supernatant and continue with the subsequent step in the protocol.
1 dpf – 10 dpf	Loose pellets	Not enough centrifugation time/ speed.	*Re*-centrifuge the sample for a minute more with 700 rcf to settle all the cells at the bottom. If even a small pellet is still loose, leave 10 µl of solution at the bottom of the eppendorf while discarding the supernatant.

Protocol validation

Using this optimized protocol, we successfully obtained high-quality single-cell sequencing data across a diverse range of developmental stages, spanning from 10 hpf up to 10 dpf [11] (data explorable at https://zebrahub.ds.czbiohub.org/transcriptomics). Having achieved single embryo resolution and a substantial sequencing depth enabled us to conduct inter-individuality studies by comparing sibling embryos at different time points during development [11] (https://zebrahub.ds.czbiohub.org/iiv?name=iiv_var).

Limitations

We report an optimized dissociation protocol for obtaining high-quality single cells from individual embryos. By tailoring our protocol to the embryos' developmental stage, we achieved improved single-cell isolation efficiency and cell yield. We implemented a stepwise optimization approach by altering the dissociation media, technique, centrifugation speed, and time. This ensured the preservation of cell integrity and viability needed for downstream applications.

Despite its versatility in accommodating the different developmental stages of zebrafish embryos, there are potential ways to improve our protocol. During mechanical digestion, for the most part, earlier time points are relatively easier to dissociate. However, we have noticed that when the embryos start forming more rigid structures such as eyes and a mature spinal cord, the embryo's cells are much more challenging to dissociate completely. To address this, we could increase the volume of collagenase in the dissociation media or use small pestles for dissociation. Another portion of the protocol worth improving is ensuring cellular debris is minimal.

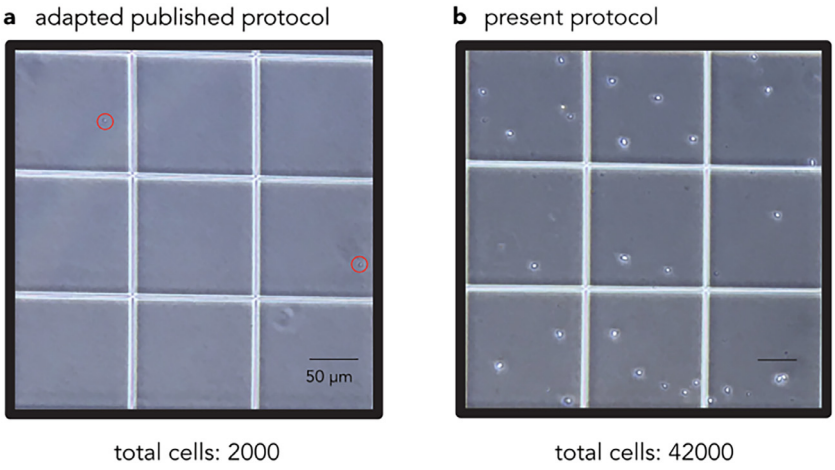

Fig. 3. Dissociation results from our protocol compared to published protocols. (a) Adapting the published protocol for pooled embryos to dissociate using an individual embryo results in a significantly low average of only 2000 cells when resuspended in 25 µl (80 cells/µl). (b) Employing our optimized protocol for individual embryo single-cell sequencing achieves an excellent yield of around 42,000 cells when resuspended in 25 µl (1680 cells/µl).

The mechanical dissociation of the embryos is breaking apart extracellular matrices, and these lysed or dead cells may cause RNA leakage (detrimental to OMICs applications). This issue is more prevalent with harsher pipetting and multiple rounds of mechanical dissociation. Adjusting the composition of the dissociation media to incorporate more collagenase or using a pestle gently to avoid multiple rounds of mechanical dissociation can also help mitigate this problem.

Implementing individual embryo single-cell resolution opens possibilities for future research and advances in multiple fields. We can delve into the complexities of development by enabling the characterization of dynamic gene expression patterns, regulatory transitions, and molecular events for individuals. Investigating single cells from individual embryos in disease models may lead to identifying novel therapeutic targets and developing personalized medicine strategies. Overall, utilizing individual embryo single-cell resolution presents abundant opportunities for future research, paving the way for significant progress in various fields.

CRediT author statement

Shruthi VijayKumar: methodology, validation, investigation, writing original draft. **Michael Borja:** validation, investigation. **Norma Neff:** conceptualization, supervision, funding acquisition. **Loïc A. Royer:** conceptualization, supervision, funding acquisition. **Merlin Lange:** conceptualization, methodology, validation, supervision, writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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