

Supplementary Information for 3D Iterative Immunofluorescence Imaging on Whole-Mount Samples

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A. General immunofluorescence protocol

Note: This section provides a general immunofluorescence protocol for testing antibody compatibility with the sample without the sample mounting step or any elution controls that are described in the main Procedure section of this protocol.

1. Prepare conventional blocking buffer (cBB) and PROTOS imaging buffer.
2. Prepare the desired number of samples as described in Procedure, Section A. For zebrafish embryos, we typically use 3–5 samples of different timepoints to test compatibility per antibody.
3. Place the embryos in an Eppendorf tube containing 100 µL of DPBS.
4. Add blocking buffer to the embryos by replacing DPBS with 100 µL of cBB. Incubate at RT for 1 h.
5. Prepare the primary antibody mixture in cBB.
 - a. Use a dilution determined before or use manufacturer recommendations given for immunohistochemistry/immunofluorescence (e.g., 1:100).

Note: We typically combine multiple primary antibodies of different host species into one experiment (e.g., one mouse, one rabbit, and one goat).

6. Wash three times with PBS-T.
7. Add the primary antibody mixture and incubate overnight at RT.
8. Wash five times with PBS-T for 10 min each at RT.
9. Prepare the secondary antibody mixture and nuclear dye in cBB.
 - a. Use a dilution determined before or use manufacturer recommendations given for immunohistochemistry/immunofluorescence (e.g., 1:200).

Note: Secondary antibodies are selected based on the primary antibody host species and the fluorescence channels of the used microscope. We typically use DAPI in 405 nm channels in combination with Alexa Fluor–coupled secondary antibodies in 488, 568, and 647 nm channels.

10. Add the secondary antibody mixture and incubate at RT for 4 h.
11. Wash five times with PBS-T for 10 min each at RT.
12. Slide-based imaging for antibody testing:
 - a. Prepare a microscopy glass slide by adding imaging spacers on top to generate a well-like structure.
 - b. Place the stained embryos with the round rise of the animal pole facing up on the glass slide.
 - c. Aspirate all liquid and replace with PROTOS imaging buffer.
 - d. Place a coverslip on top of the sample and the imaging spacer to seal.
 - e. Incubate the samples for at least 20 min for RI-matching before imaging.
 - f. Acquire fluorescence images as desired.

Note: Determine if the staining has worked by comparing it with the expected pattern based on references. If needed, adjust the dilutions for an improved signal-to-noise ratio. We provide a list of working antibodies for zebrafish embryos in Table S1, with corresponding example images in Figure S1.

B. List of antibodies tested and compatible with ZF embryos

Available via Table S1 (supp_table_ZF_working_antibodies.xlsx). Example images provided in Figure S1.

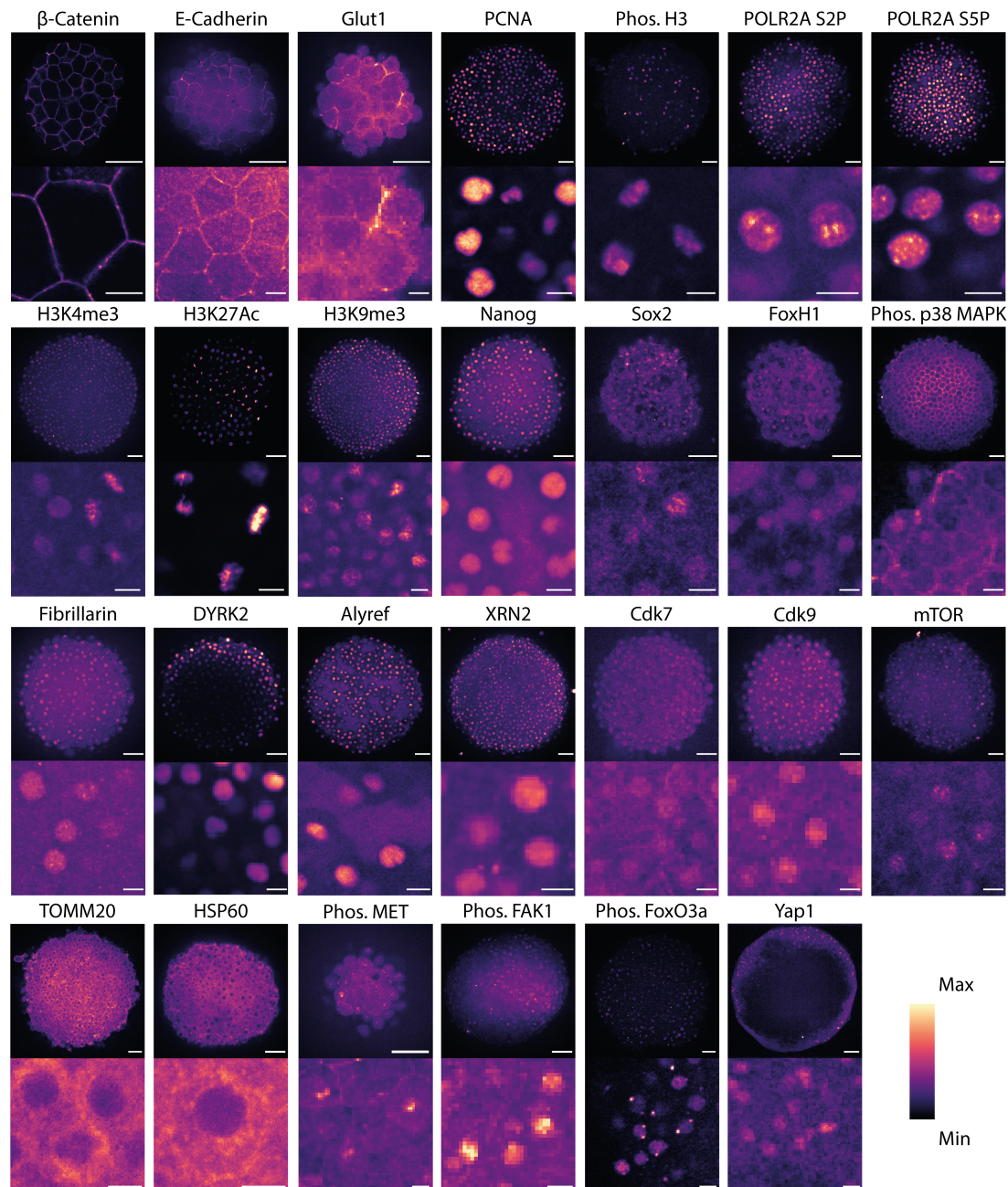


Figure S1. Example immunofluorescence images in zebrafish embryos. Example center z-slices of the full zebrafish embryo (top rows) and zoomed-in images (bottom rows) of selected antibodies compatible with the zebrafish embryo. Details about the antibodies and the dilutions are provided in Table S1. The LUT is the Napari colormap “magma.” Scale bars correspond to 75 μ m in the full embryo images (top rows) and 7.5 μ m in the corresponding zoomed images (bottom rows).

C. Antibody validation and elution controls

Note: This section describes how to validate antibody compatibility with the selected sample and the 3D-4i method and determine the optimal staining cycle. For additional guidance, consult the General notes below and those of the original 4i protocol [6].

1. Antibody validation:

- Perform the sample preparation and plate mounting as described in Procedure, Sections A and B.
- Skip the elution step and perform a classic immunofluorescence staining with primary and secondary antibody steps (Supplementary Section A). Select primary and secondary dilution factors based on prior knowledge or the manufacturer's recommendations (for immunohistochemistry/immunofluorescence).
- Clear the sample with the RI-matching medium of choice and image the sample with the same settings planned to be used during 3D-4i.
- Compare the signal to reference stainings and published papers/protocols.
- If the expected staining pattern is obtained, the antibody is compatible with the sample and the selected clearing method. Its compatibility with the 3D-4i method can then be assessed (Supplementary Section C, step 2).

Note: Start with a low antibody concentration and increase as necessary. If required, choose another RI-matching solution (see General notes).

- If the pattern of the staining shows staining bias (see Figure S2), optimize the antibody concentration or exchange the secondary antibody step with a 1-step staining approach, as described in Supplementary Section D. Please also consider the Troubleshooting section.
- A selection of tested antibodies compatible with the 3D-4i protocol is provided in Table S1, with example images for their staining pattern presented in Figure S1.

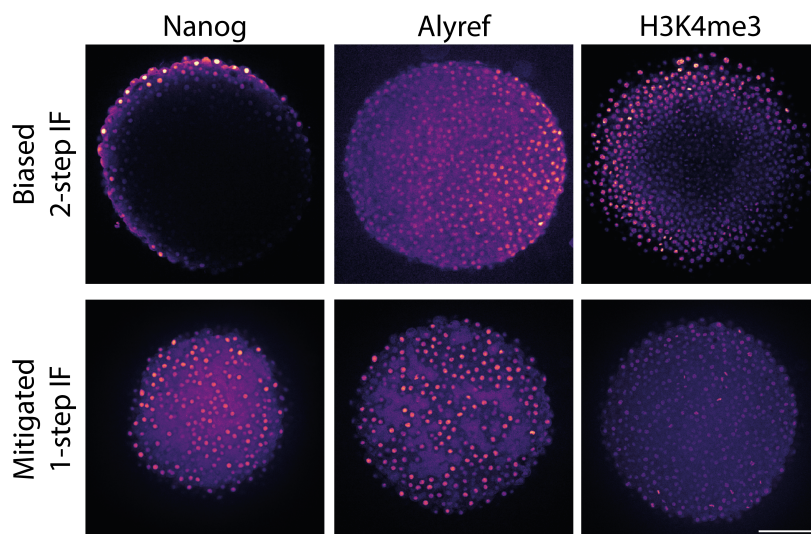


Figure S2. Staining bias mitigation using directly coupled primary antibodies. Top row: Biased two-step immunofluorescence staining of Nanog, Alyref, and H3K4me3 in a 3.5-hpf-old zebrafish embryo using primary and secondary antibodies. Bottom row: Mitigation of observed bias by using Fab fragment-coupled primary antibodies in a 1-step immunofluorescence staining. For further information, refer to Supplementary Section D. The LUT is the Napari colormap “magma.” Scale bar: 25 μ m. IF: Immunofluorescence.

2. Antibody elution and epitope retention tests:

- Perform the sample preparation and plate mounting as described in Procedure Sections A and B.
- Prepare at least three wells containing multiple samples each (e.g., 5 samples per well to account for variability/losses) corresponding to three conditions:
 - Control well A: to see the compatibility with the elution buffer.
 - Control well B: to test epitope retention after four elution cycles.
 - Control well C: to examine the elution efficiency for a given antibody.

Note: See Figure S3A for the flowcharts of the individual controls.

- c. Control well A: Perform a complete 3D-4i cycle, including the pre-elution step, and image the sample.
 - i. This will define whether the antibody is compatible with the elution step and serve as reference staining.
 - ii. After the first imaging, elute the sample again and re-stain by only adding secondary antibodies. Compare the fluorescence intensity with the initial imaging.
 - iii. This will define whether the primary antibody was successfully/sufficiently removed by one elution step. In case the signal is still detectable, perform Control C.
 - iv. A selection of tested antibodies compatible with the 3D-4i protocol, with example images for what elution of the signal can be expected, is presented in Figure S3B.

- d. Control well B: Perform four “mock” 3D-4i cycles, running the whole protocol as is, but omitting the addition of primary and secondary antibodies and the clearing step (elution, washing, blocking sBB, washing, cBB, washing, cBB, washing). Then, perform a complete 3D-4i cycle with the antibody staining and clearing, and image the sample. This control checks if the antigenicity of the epitope is maintained or lost with increased cycle number.

Note: Signals of highly abundant epitopes, such as the cytoskeleton, are maintained better, while signals of low-abundance epitopes, such as phosphorylated proteins, can be lost in later cycles.

- e. Control well C: Perform a complete 3D-4i cycle with the initial antibody staining and image the sample. Then, add more “mock” 3D-4i cycles, only adding secondary antibodies again and the optical clearing step, and image the sample at the end of each mock cycle. Check if the detectable fluorescence signal drops with added elution steps. This control checks whether the signal of the immunofluorescence staining can be eluted efficiently.

Note: Signals of highly abundant epitopes, such as the cytoskeleton, are harder to elute, while signals of low-abundance epitopes, such as phosphorylated proteins, easily elute.

- f. For all three controls, in addition to visual inspection of the retained signal, perform a quantitative analysis using FIJI, as described in the Data analysis section titled “Basic analysis for antibody panel design (FIJI).”
- g. After confirming antibody compatibility and elutability using the appropriate controls, determine the optimal staining cycle for each epitope. Balance signal strength against elution efficiency when assigning antibodies to earlier or later cycles. See Figure S3B for examples of elution quality after a single elution cycle, and Table 1 in Procedure Section C for an example of antibody panel design and cycle allocation.

- h. If a high residual signal is still present after performing the elutions, controls, and adjusting the staining, consider the Troubleshooting section.

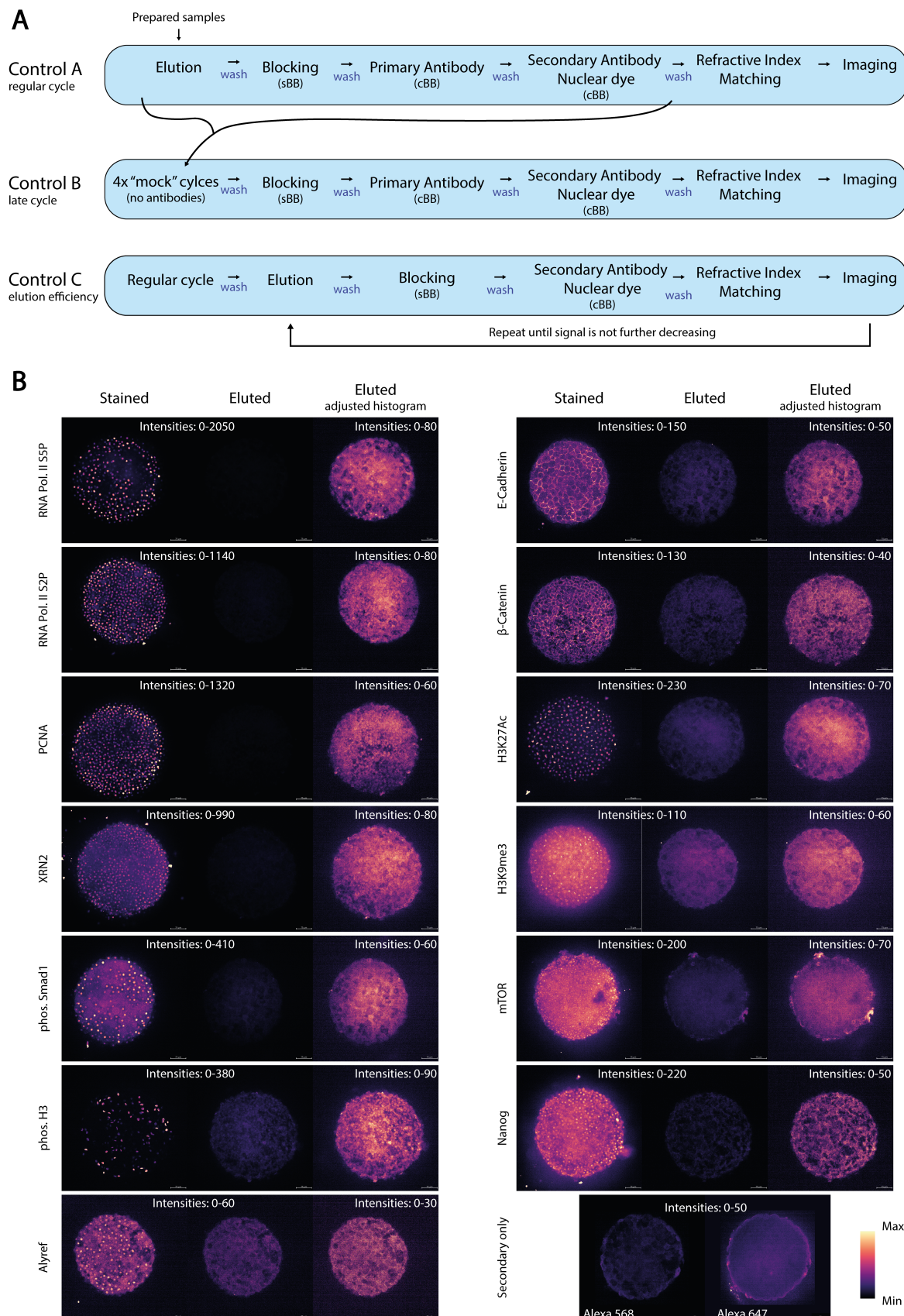


Figure S3. Antibody compatibility evaluation and elution controls. (A) Schematics of Control A: Single elution regular 3D-4i cycle; Control B: Late cycle compatibility; Control C: Signal elution efficiency. Described in detail in Supplementary Section C, step 2. (B) Example stainings of antibodies and corresponding secondary re-staining after elution to check for

residual signal. The first two images show the antibody staining and the respective elution control with the same display intensity range. The third image of each antibody shows the elution control rescaled to see the pattern of the residual signal. Sorted by their initial signal strength vs. signal retention after single elution (left: high signal to residual intensity; right: lower initial signal and therefore higher relative residual signal). The intensities of the histogram have been adjusted (depicted for each image in white), so the residual signal can be better seen. Without intensity rescaling, the eluted signal is difficult to evaluate. It is important to not only check the residual signal intensity but also the pattern of eventual residual signal (e.g., nuclear signal still present in an epitope re-staining that is expressed inside the nucleus may be problematic for further stainings of nuclear epitopes in later cycles in the same fluorescence channel). For comparison, we also provide two example images of an unspecific signal to expect in the absence of a primary antibody. Secondary antibody with an Alexa Fluor 568 coupling (donkey anti-mouse) and Alexa Fluor 647 coupling (donkey anti-rabbit). AB: antibody, sBB: 3D-4i blocking buffer, cBB: conventional blocking buffer. The LUT is the Napari colormap “magma.” Scale bars: 75 μ m.

D. Mitigation of staining bias using Fab fragments

Note: This section provides an additional step in the immunofluorescence protocol for antibodies that were tested to work with the sample but show staining bias, decreasing toward the center of the sample. To mitigate the bias, fluorescently tagged Fab fragments are pre-incubated with the primary antibody and added as a 1-step staining afterward. The secondary antibody addition is then omitted for this fluorescence channel. Zenon™ IgG labeling kits (Invitrogen) have been successfully tested to be compatible with the 3D-4i platform; other Fab fragments or nanobodies are likely also compatible; we recommend testing elutability.

Critical: As the Fab fragments already carry fluorophores, make sure to protect the solutions prepared and the sample from light at all times to minimize bleaching (see General notes).

Using the Zenon™ IgG labeling kit (Invitrogen) for primary antibody coupling:

1. Choose the matching target species for the primary antibody to be coupled and select the channel of the Fab fragment-linked fluorophore to be compatible with the other epitopes in the cycle to be stained and compatible with the fluorescence cubes of the used microscope.
2. Prepare 1 μ g of target primary antibody in a fresh tube and add 5 μ L of Zenon Fab fragment solution. Incubate at RT for 5 min.
3. Bind surplus unbound Fab fragments by adding 5 μ L of the corresponding species blocking reagent, and incubate for another 5 min at RT.
4. Prepare the Fab-fragment-coupled primary antibody as described in Procedure Section C, at 2 $\times$ the desired final concentration in 2 $\times$ cBB.
5. Please refer to the most up-to-date version of the manufacturer's protocol (<https://www.thermofisher.com/ch/en/home/references/molecular-probes-the-handbook/antibodies-avidins-lectins-and-related-products/zenon-technology-versatile-reagents-for-immunolabeling.html>).
6. For example mitigations, see Figure S2. For an evaluation of the mitigation, see Figure S4. Here, we confirmed the mitigation of staining bias by staining for endogenously expressed H2A-mCherry using a standard 2-step immunofluorescence protocol and comparing a 1-step immunofluorescence protocol with Fab fragments.

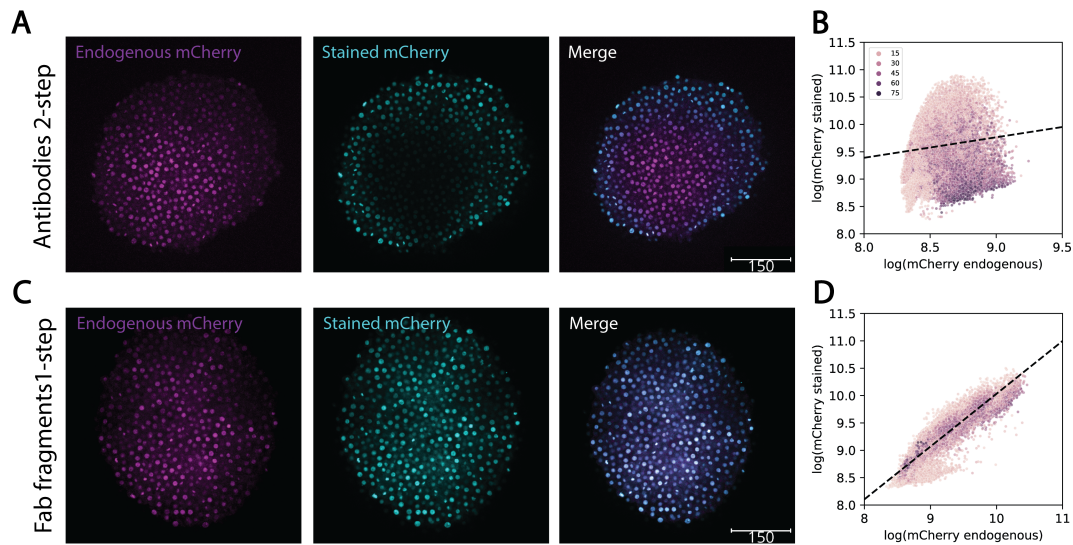


Figure S4. Evaluation of staining bias mitigation using Fab fragment 1-step immunofluorescence. (A) Example center z-slice of an embryo expressing endogenous H2A-mCherry, 2-step immunofluorescence against mCherry epitope, and merge. (B) Quantification of signal correlation from endogenous and IF; hue represents distance from embryo border in μm ; dashed line shows linear regression. Each dot is a single cell of the embryo. $n = 6$ embryo replicates. Center nuclei clearly show less signal from staining than there should be, as seen with the endogenous expression. (C) Same as in A, but using Zenon Fab-fragments. (D) Same quantification as in B, but using Zenon Fab-fragments. The signal of endogenously expressed reporter and staining correspond much better in the 1-step staining than the 2-step staining approach. Scale bars: 150 μm .

E. (Optional) Liquid handling platform

Note: To scale the protocol to high-throughput, it is highly recommended to set it up using an automated liquid handling platform to minimize hands-on time and maximize sample retention.

1. To ensure adherence of the mounted samples, all pipetting steps of the 3D-4i protocol can be carried out using an automated liquid handling platform. The following protocols facilitate the steps in Procedure Section C and Supplementary Section C.
 - a. Elution (including washing).
 - b. DPBS wash.
 - c. Dispensing of blocking buffer/antibody mixture.
 - d. Dispensing of imaging buffer.
 - e. Washout of imaging buffer.
2. An example setup of the platform is displayed in Figure S5A/B. In one experiment, we performed multiplexing on 212 embryos (209 are shown in Figure S5C).
3. All protocols and pipetting settings are displayed in Tables S2 and S3. Use the slow setting for dispensing and washing of the RI buffer and regular settings for all other steps.

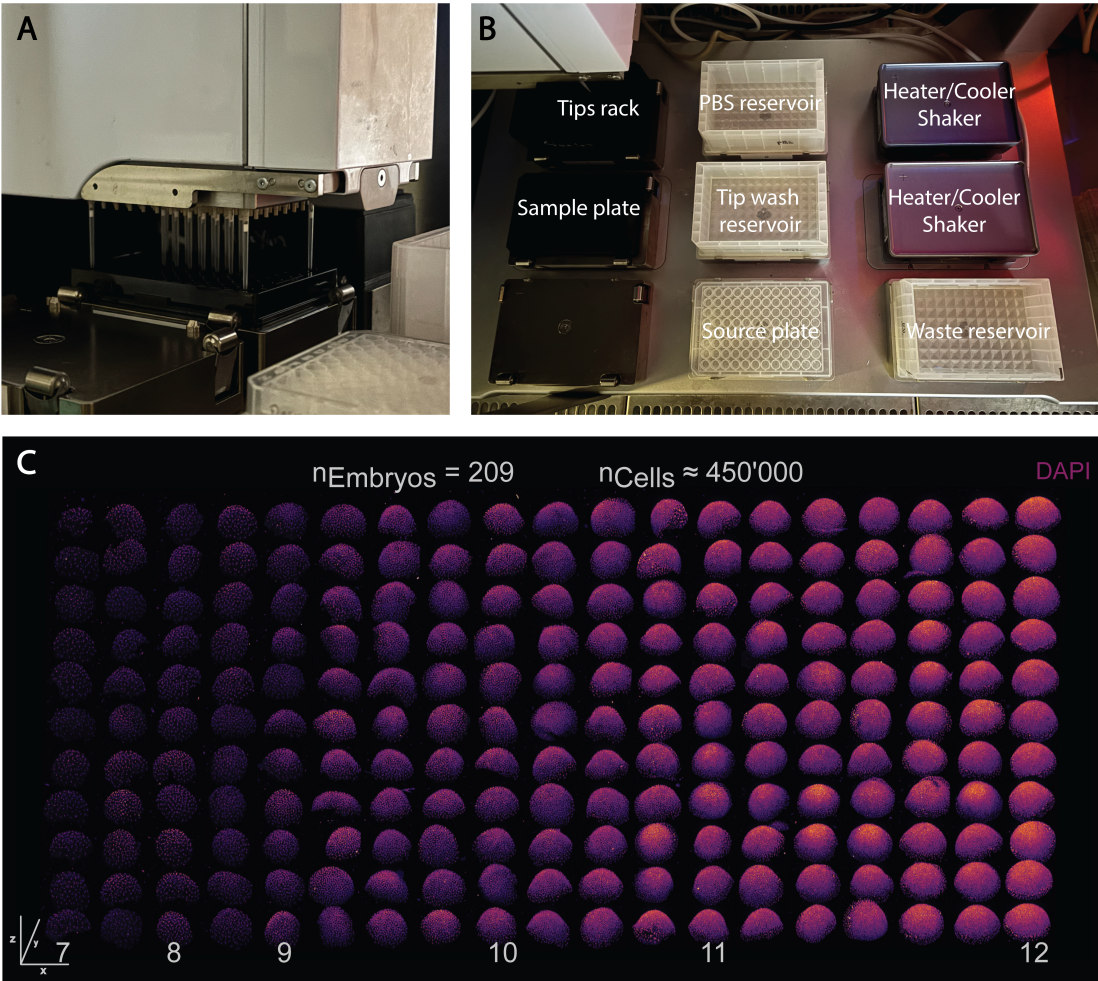


Figure S5. Automated liquid handling platform enables high-throughput 3D-4i multiplexing. (A) Image of an example platform compatible with the protocol (BRAVO, Agilent Technologies). Using a pipetting head compatible with a 96- and 384-well plate format and 10 and 70 μ L pipette tips. Assay plates used are in the 96-well format, while tips are stored in 384-well racks. (B) Example layout of the platform deck containing a sample plate (target), the source plate for blocking buffer, antibody mixture, and clearing reagent dispensing, the waste reservoir, the DPBS reservoir, the tip wash reservoir, and pipette tip rack positions. (C) Overview of 209 zebrafish embryos DAPI-stained from a high-throughput 3D-4i experiment. Sorted from left to right by total nuclei count. Cell division cycles are marked at the bottom as a proxy for age (7–12). Panel C is adapted from [10]. The LUT is the Napari colormap “magma.” x, y, z scale bars, 650 μ m.

Table S2. Pipetting settings to operate protocols on the BRAVO automated liquid handling platform. Well bottom distance is adjusted to prevent embryo detachment. Regular settings are compatible with all protocols displayed in Table S3, except for dispensing and washing of PROTOS imaging buffer, which uses the slow settings. Aspiration and dispensing of liquids are performed with the same settings.

Pipetting settings	Regular	Slow
Well bottom distance (mm)	2.5	2.5
Velocity (μ L/s)	10	5
Acceleration (μ L/s ²)	20	10
Post-aspirate/dispense delay (ms)	0	100
Z-axis parameters		
Velocity into wells (mm/s)	10	10
Acceleration into wells (mm/s ²)	20	20
Velocity out of wells (mm/s)	10	10
Acceleration out of wells (mm/s ²)	20	20

Table S3. Protocols for automated liquid handling. 96-well plates are operated and placed in the respective position of the 3D-4i assay plate and plates containing DPBS, antibody solution, blocking buffer, elution buffer, and/or imaging buffer (see Figure S5B). Individual actions and respective loops are indicated with their pipetting settings. Mix pos1/2 refers to pipetting 50 μ L up and down (4 $\times$) in the upper-left well quadrant (pos1) and lower-right quadrant (pos2). AB, antibody; BB, blocking buffer; EB, elution buffer; IB, imaging buffer.

Protocol DPBS wash		
Duration 40 min		
Protocol		
Loop	Action	Plate
	Tips on	
2 $\times$	Aspirate 70 μ L	3D-4i plate
	Dispense waste	Waste
12 $\times$	Aspirate 70 μ L	DPBS plate
	Dispense DPBS	3D-4i plate
	Mix 50 μ L pos1	3D-4i plate
	Mix 50 μ L pos2	3D-4i plate
	Aspirate 70 μ L	3D-4i plate
	Dispense waste	Waste
	Tips off	

Protocol Dispense antibodies /blocking buffer		
Duration 10 min		
Protocol		
Loop	Action	Plate
	Tips on	
2 $\times$	Aspirate 70 μ L	3D-4i plate
	Dispense waste	Waste
	Aspirate 70 μ L	AB/BB plate
	Dispense AB/BB	3D-4i plate
	Mix 50 μ L pos1	3D-4i plate
	Mix 50 μ L pos2	3D-4i plate
	Tips off	

Protocol Dispense the imaging buffer		
Duration 20 min		
Protocol		
Loop	Action	Plate
	Tips on	
2 $\times$	Aspirate 70 μ L	3D-4i plate
	Dispense waste	Waste
2 $\times$ (slow settings), 3 $\times$ (regular settings)	Aspirate 60 μ L	IB plate
	Dispense	3D-4i plate
	Mix 50 μ L pos1	3D-4i plate
	Mix 50 μ L pos2	3D-4i plate
	Aspirate 60 μ L	3D-4i plate
	Dispense waste	Waste
	Tips off	

Protocol Elution		
Duration 60 min		
Protocol		
Loop	Action	Plate
	Tips on	
2 $\times$	Aspirate 70 μ L	3D-4i plate
	Dispense waste	Waste
4 $\times$	Aspirate 70 μ L	EB plate
	Dispense EB	3D-4i plate
	Mix 50 μ L pos1	3D-4i plate
	Mix 50 μ L pos2	3D-4i plate
	Aspirate 70 μ L	3D-4i plate
	Dispense waste	Waste
12 $\times$	Aspirate 70 μ L	DPBS plate
	Dispense DPBS	3D-4i plate
	Mix 50 μ L pos1	3D-4i plate
	Mix 50 μ L pos2	3D-4i plate
	Aspirate 70 μ L	3D-4i plate
	Dispense waste	Waste
	Tips off	

Protocol Washout of the imaging buffer		
Duration 60 min		
Protocol		
Loop	Action	Plate
	Tips on	
	Aspirate 70 μ L	3D-4i plate
	Dispense waste	Waste
4 $\times$ (slow settings), 8 $\times$ regular settings	Aspirate 65 μ L	DPBS plate
	Dispense	3D-4i plate
	Mix 50 μ L pos1	3D-4i plate
	Mix 50 μ L pos2	3D-4i plate
	Aspirate 65 μ L	3D-4i plate
	Dispense waste	Waste
	Tips off	