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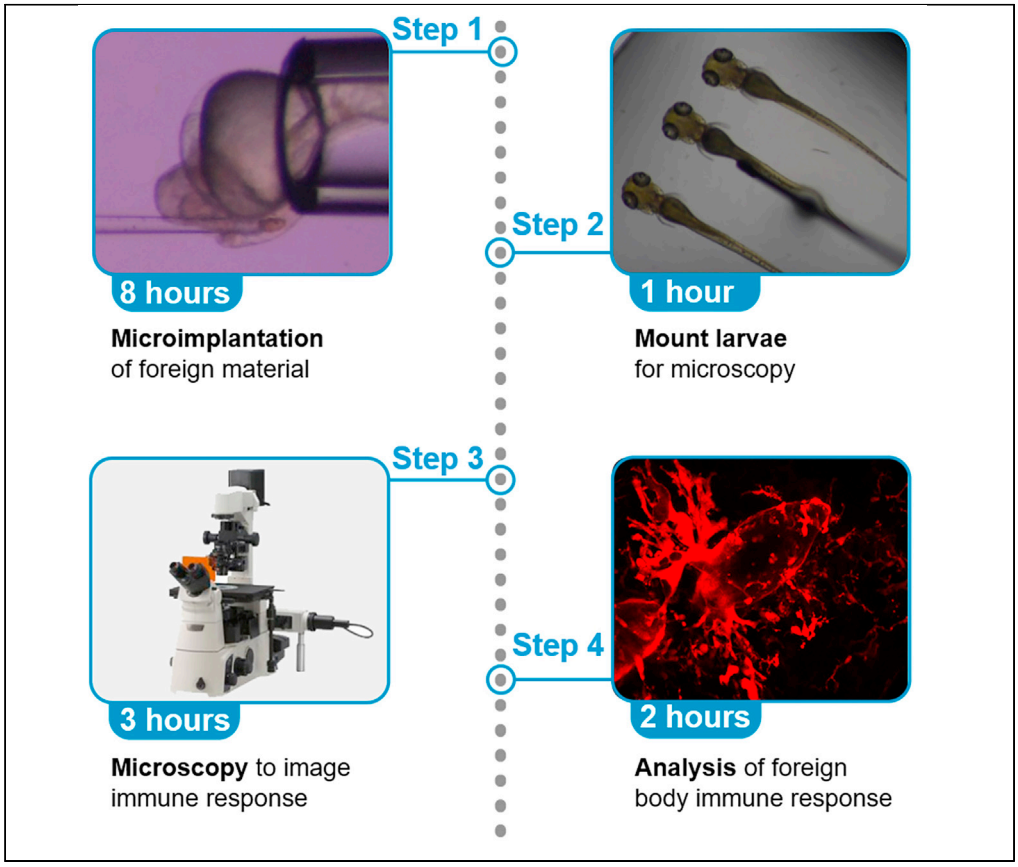
Protocol

Microimplantation of foreign materials for assessment of foreign body immune responses and granuloma formation in zebrafish larvae



This protocol describes the microimplantation of foreign materials such as schistosome eggs, polymer beads, and other microscopic objects into the small and optically transparent larval zebrafish for the assessment of immune responses, including granuloma formation. This protocol has wide applicability for both fundamental studies on host responses to parasite eggs and other foreign bodies, as well as the testing of potential biomaterials and devices used for human medical implants.

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Highlights

Foreign body immune responses are critical with both pathogenesis and medical implants

This protocol details the microimplantation of foreign materials into zebrafish larvae

After implantation, foreign body immune responses are observed by intravital microscopy

Potential use in testing biomaterials and devices used for human medical implants

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Microimplantation of foreign materials for assessment of foreign body immune responses and granuloma formation in zebrafish larvae

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SUMMARY

This protocol describes the microimplantation of foreign materials such as schistosome eggs, polymer beads, and other microscopic objects into the small and optically transparent larval zebrafish for the assessment of immune responses, including granuloma formation. This protocol has wide applicability for both fundamental studies on host responses to parasite eggs and other foreign bodies, as well as the testing of potential biomaterials and devices used for human medical implants.

For complete details on the use and execution of this protocol, please refer to Takaki et al. (2021a) and (2021b).

BEFORE YOU BEGIN

Assemble the vacuum-assisted MicroProbe (VAMP)

Timing: 2–3 h

⚠ CRITICAL: Ensure that power supply is disconnected before modifying the aquarium pump. Use safe handling of tools.

1. Reverse the flow of the aquarium pump (Methods video S1):
   a. Ensure pump is disconnected from power supply.
   b. Remove the two screws on the bottom of the aquarium pump.
   c. Remove the housing.
   d. Remove the pump mechanism, rotate 180°, and then place back into housing.
   e. Reseal the rubber gasket.
   f. Reassemble the housing and re-secure the screws.
   g. Plug in the power supply and verify that the valve now produces suction.
2. Prepare the microprobe (Methods video S2):
   a. Disassemble a standard ballpoint stick pen and discard the ink reservoir and the metal ballpoint tip using pliers.
   b. Pass tissue paper through the bore of the plastic pen tip to remove residual ink.
   c. Immerse the pen tip in 5 mL 70% ethanol in a 15 mL centrifuge tube and agitate to remove additional ink.
   d. Reassemble the pen tip.
   e. Drill a 4.5 mm hole 40 mm from the end.

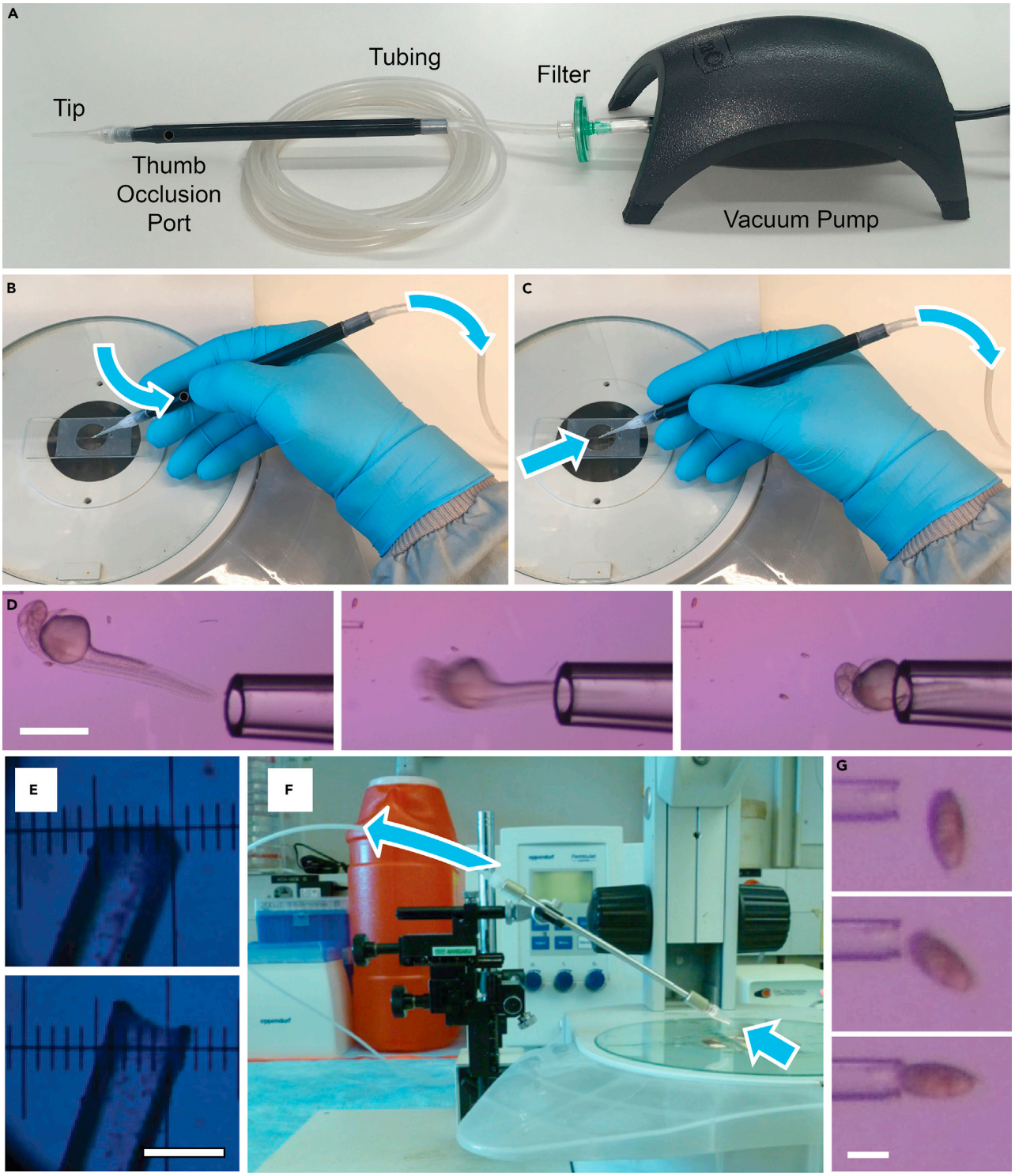

Figure 1. VAMP and CAIN components and function

(A) Vacuum-Assisted MicroProbe (VAMP) components as assembled following Methods videos S1 and S2.

(B–D) VAMP in use. (B) Air flows through the thumb occlusion port when open. (C) When the thumb occlusion port is closed, suction is transferred to the tip which allows for (D) grasping of the zebrafish larva.

Figure 1. *Continued*
(E–G) Capillary-Assisted Implantation Needle (CAIN) and its usage. (E) The CAIN is double-beveled (upper image) so at to produce a sharp point, while also producing a nest for stable grasping of objects to be implanted (rotated 90°, lower image). (F) The CAIN is attached to a micromanipulator for control along the X,Y, and Z axes. (G) Capillary action results in grasping of small objects.
Scale bars, 500 μm (D) and 50 μm (E and G).

f. Attach a P10 pipet tip to the end and secure by wrapping with parafilm.
g. Remove 5 mm of material from the base end of the pen by cutting with a razor.
h. Attach 0.5 cm diameter silicone tubing to the base of the microprobe and secure with parafilm.

3. Assemble the VAMP (Methods video S2 and Figure 1A):
   a. Attach a 0.2 μm syringe filter to the pump input.
   b. Attach the lose end of the silicone tubing to the syringe filter.

Create the capillary-assisted implantation needle (CAIN)

Timing: 1–2 h

4. Pull the borosilicate capillaries using Sutter Instruments P-2000 micropipette puller with the following settings: Heat = 350, FIL = 4, VEL = 50, DEL = 225, PUL = 150.
5. Break the needle to an outer diameter of approximately 50 μm using jeweler's forceps.
6. Grind the needle to a beveled point using a microgrinder. Rotate the needle 180° and then repeat to create a double-beveled needle.

⚠ CRITICAL: Ensure that power supply is disconnected before modifying the aquarium pump. Use safe handling of tools.

Optional: The modification and use of an aquarium pump in Step 1 can be skipped if a laboratory vacuum inlet is available. In this case, the tubing can be attached to the 0.2 μm filter, and then attached to the supplied vacuum inlet without the need of a mechanical pump.

Note: The outer diameter of the CAIN (**Step 5** and Figure 1) is optimized for the implantation of materials of ~30–60 μm diameter, and may be reduced or increased to accommodate materials of differing sizes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Schistosoma mansoni eggs Puerto Rican strain	Gabriel Rinaldi	N/A
Chemicals, peptides, and recombinant proteins		
Instant Ocean	Spectrum Brands	N/A
PTU (1-phenyl-2-thiourea)	Sigma-Aldrich	A5040
Tricaine	Sigma	A5040
Low Melting Point (LMP) Agarose	Thermo Fisher	R0801
Experimental models: organisms/strains		
Zebrafish (*Danio rerio*): wild type AB strain male and female larvae at 30–48 h post-fertilization	Zebrafish International Resource Center	ZDB-GENO-960809-7
Zebrafish: *Tg(mpeg1:Brainbow)*^{w201} male and female larvae at 30–48 h post-fertilization	Pagán et al., 2015	ZDB-FISH-151204-7
Software and algorithms		
NIS-Elements 4 (version 5.21.01)	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-advanced-research

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Ballpoint stick pen	Papermate	33311
3/16" Drill bit and drill	n/a	n/a
Micropipette tips	PakRak	53503–816
Parafilm	Fisher Scientific	11772644
Aquarium pump	Tetra	APS50
Acrodisc 0.2 μm syringe filter	Pall Laboratories	514–4126
Silicone tubing 0.5 cm × 1 m	Fisher Scientific	TSR0100200P
Borosilicate glass capillaries with thin wall with filament (1.0 mm OD × 0.78 mm ID × 10 cm length)	Harvard Instruments	Gc100Tf-10
Micropipette puller	Sutter Instruments	P-2000
Jeweler's no. 5 forceps	VWR Scientific	21909–464
Microgrinder	Warner Instruments	64-1617 Mfg-5Ae
Pipette pump	Bel-Art Products	378980000
Wide-bore Pasteur pipette	Kimble Chase	63A53WT
Small petri plate 55 mm	Fisher Scientific	11739242
Standard petri plate, 100 mm	Falcon	351029
Incubator, 28°C, Isotemp	Fisher Scientific	11–690-637D
Dissecting microscope	Nikon	SMZ-800
Microinjector system	Eppendorf	Femtojet Express
Micromanipulator	Narishige	M-152
Iron baseplate for micromanipulator	Narishige	IP
Depression slide	VWR Scientific	48324–001
Optical glass-bottom plates, 6-well	MatTek Corporation	P06G-1.5-20-F
Nikon A1 confocal laser microscope	Nikon	A1

MATERIALS AND EQUIPMENT

Fish Water		
Reagent	Final concentration	Amount
Instant Ocean	0.18 g/l Instant Ocean	3.6 g
ddH_2O	n/a	20 L
Total	**n/a**	**20 L**

Note: Dissolve 3.6 grams of Instant Ocean in 20 L of ddH_2O. Store at room temperature (22°C) for up to 3 months.

PTU stock solution (20×)		
Reagent	Final concentration	Amount
PTU	20×	0.3 g
ddH_2O	n/a	500 mL
Total	**n/a**	**500 mL**

Note: Dissolve 0.3 g of PTU powder in 500 ml of dH_2O using low heat and constant stirring. PTU may take several hours to dissolve. Filter-sterilize the solution and store it up to 1 month at 4°C.

⚠ CRITICAL: This solution is highly toxic and skin sensitizing. Wear appropriate protective clothing and avoid ingestion, inhalation or contact with skin or eyes.

Tricaine stock solution		
Reagent	Final concentration	Amount
Tricaine powder	n/a	0.4 g
ddH_2O	n/a	100 mL
0.5 M $NaHCO_3$	n/a	to pH 7.0
Total	n/a	**100 mL**

Note: Dissolve 0.4 g of tricaine (3-amino benzoic acid ethyl ester) in 100 ml of dH_2O. Adjust the pH to 7.0 with 0.5 M $NaHCO_3$. Store the solution for up to 1 month at 4°C.

⚠ CRITICAL: Tricaine is an irritant. Wear appropriate protective clothing and avoid ingestion, inhalation or contact with skin or eyes.

Fish Water with PTU		
Reagent	Final concentration	Amount
PTU stock solution (20×)	1×	50 mL
ddH_2O	n/a	1 L
Total	**n/a**	**1 L**

Note: Store at room temperature for up to 2 weeks.

LMP agarose stock		
Reagent	Final concentration	Amount
LMP powder	$1.5\%^{w/v}$	1.5 g
Fish water	n/a	100 mL
Total	**n/a**	**100 mL**

Note: Combine 1.5 g of LMP agarose to 100 mL of fish water and then microwave until dissolved. Aliquot into 1.7 mL microcentrifuge tubes and store in a heat block at 43°C for up to 3 weeks.

STEP-BY-STEP METHOD DETAILS

Microimplantation of foreign material

Timing: 4–8 hours

In the following steps, larval zebrafish will be anesthetized and then surgically microimplanted with small foreign materials such as beads and schistosome eggs.

1. Anesthetize larval zebrafish at 30–48 h post-fertilization by immersion in $0.02\%^{w/v}$ tricaine.
   a. Add 630 μL of $0.4\%^{w/v}$ tricaine to 10 mL of fish water in a small 55 mm petri plate.
   b. Using a wide-bore Pasteur pipette, transfer ≤100 larvae to the fish water containing tricaine. Incubate at room temperature for 3 min.
   c. Transfer 10–20 larvae in approximately 1 mL of fish water containing tricaine to a depression slide. To reduce clutter, use the microprobe to group the larvae together at the nearest edge of the depression.
2. Implant larvae with foreign material (Methods video S3 and Figure 2).
   a. Using the VAMP, grasp a single larva by its tail. (See Troubleshooting problem 1.)

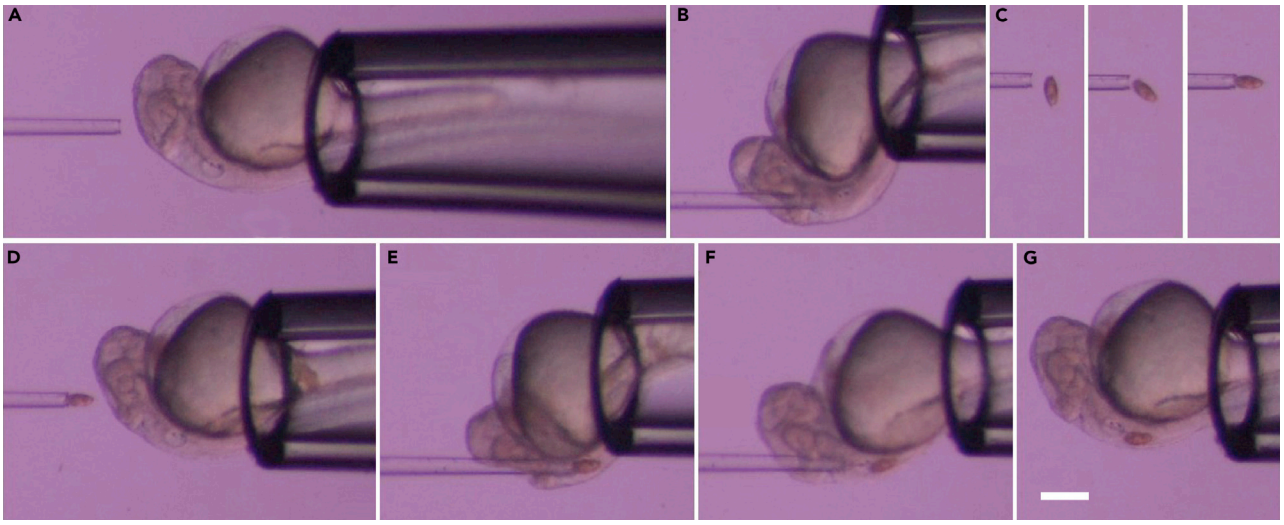

Figure 2. Procedure for implantation of foreign bodies

(A) Larva grasped using VAMP and aligned with the CAIN.
(B) Incision made through forebrain and guided into hindbrain ventricle.
(C) Egg being grasped by the CAIN.
(D) Egg aligned with the incision.
(E) Egg passed through incision and into hindbrain ventricle.
(F) Egg deposited in hindbrain ventricle.
(G) Completed implantation.
Scale bar, 150 μm. See also Methods video S3.

b. Carefully push the forebrain of the larva into the CAIN to make an incision (see Troubleshooting problem 2).
c. Remove the larva from the CAIN by gently pulling with the VAMP.
d. Position the CAIN directly above the object to be implanted (e.g., schistosome egg, polymer bead, etc.). Lower the CAIN to the object, which will then be grasped by the CAIN via capillary action.
e. Align the object with the incision.
f. Pass the object through the forebrain incision and into the hindbrain ventricle.
g. Slightly angle the VAMP relative to the CAIN to wipe the object from the tip of the CAIN, depositing it into the hindbrain ventricle.
h. Repeat steps a-g until the desired number of larvae have been implanted.
i. Transfer implanted larvae into standard 100 mm petri plates containing 35 mL of fish water containing 1× PTU. House the larvae at a density no greater than 60 larvae per plate.
j. Incubate at 28°C for the desired amount of time before analysis, typically 1 h or up to 5 days.

⚠ CRITICAL: To avoid aspirating the larva during implantation, do not use excessive thumb pressure when grasping with the VAMP (see Troubleshooting problem 1).

Optional: This procedure may be repeated to implant more than one foreign body per larva, as well as co-implantation of two or more different materials to observe their interaction with each other and with the host.

While the acellular hindbrain ventricle is ideal for assessing immune responses to foreign materials, this procedure has been used successfully to implant materials into other anatomical regions of the zebrafish, including various regions of the brain, and the yolk sac. The avascular regions of the yolk sac may be used to study angiogenesis in response to implanted materials, and the brain may be

used to study the interaction of neurons with implanted materials, for example, in the study of brain machine interfaces.

Imaging foreign body immune responses and granuloma formation

Timing: 2–4 hours

In the following steps, the previously implanted larvae will be anesthetized, mounted onto glass slides, and then imaged by microscopy.

3. Anesthetize larval zebrafish at the desired time point post-implantation by immersion in 0.016%$^{w/v}$ tricaine.
   a. Add 420 μL of 0.4%$^{w/v}$ tricaine to 10 mL of fish water in a 55 mm petri plate.
   b. Using a wide-bore Pasteur pipette, transfer ≤100 larvae to the fish water containing tricaine.
4. Mount anesthetized larvae in 1.5% low melting point agarose (Methods video S4).
   a. Transfer 3–6 anesthetized larvae in approximately 20 μL of fish water to an optical glass-bottom plate with 20 mm glass diameter wells.
   b. Add 20 μL of 1.5% low melting point agarose.
   c. Working quickly, use the microprobe to swirl the larvae to mix the agar and fish water together, then position the larvae laterally in a row, and then rotate the larvae to orient them with their hindbrain ventricle in contact with the bottom of the glass. If any larvae rotate out of position, use the probe to quickly reposition them until the agar has solidified. Return the low melting point agarose to 43°C heat block.
   d. Repeat a-c until each well contains up to 36 mounted larvae.
   e. Add 20 μL of fish water containing tricaine per group of 3–6 mounted larvae to prevent dehydration. If the imaging session will exceed 1 h, fill the bottom of the well with fish water containing tricaine to submerge the agar-mounted larvae and prevent dehydration.
5. Image implanted zebrafish by confocal microscopy
   a. Mount the plate of implanted zebrafish onto the stage of a confocal microscope.
   b. By eye, set the X and Y coordinates for each larval hindbrain ventricle, with the implanted object in the center of the field of view.
   c. Under fluorescence, set the z position to 10 μm beyond the edge of each implanted object.
   d. Set z-stack to scan through the depth of the implanted object, with an additional 10 μm beyond.
   e. Set the imaging parameters for the resolution required for either timelapse or static confocal imaging.
   f. Click the "Run" button to perform batch imaging in which each larva is automatically imaged, in sequence.
   g. Analyze each image or timelapse video as required for the specific experiment.

EXPECTED OUTCOMES

This protocol is designed to implant foreign bodies into the hindbrain ventricle of zebrafish larvae (Figures 3A and 3B). The implantation procedure itself is exceptionally well-tolerated, with no increase in mortality; larvae implanted with either one or two schistosome eggs had a survival rate of 98%–100% at 5 days post-implantation (n = 50 per group), and no discernable detriment to larval health or behavior was observed up to 12 days following implantation of schistosome eggs and polymer beads. With this high baseline of survival, potential effects on mortality and larval health may be observed when implanting new materials, and should be assessed as an experimental parameter.

Following the implantation of a foreign body into the hindbrain ventricle of transgenic zebrafish with fluorescent macrophages, the immune response may be assessed beginning as early as 1 h post-implantation, or the minimal amount of time required to mount and image the larvae after

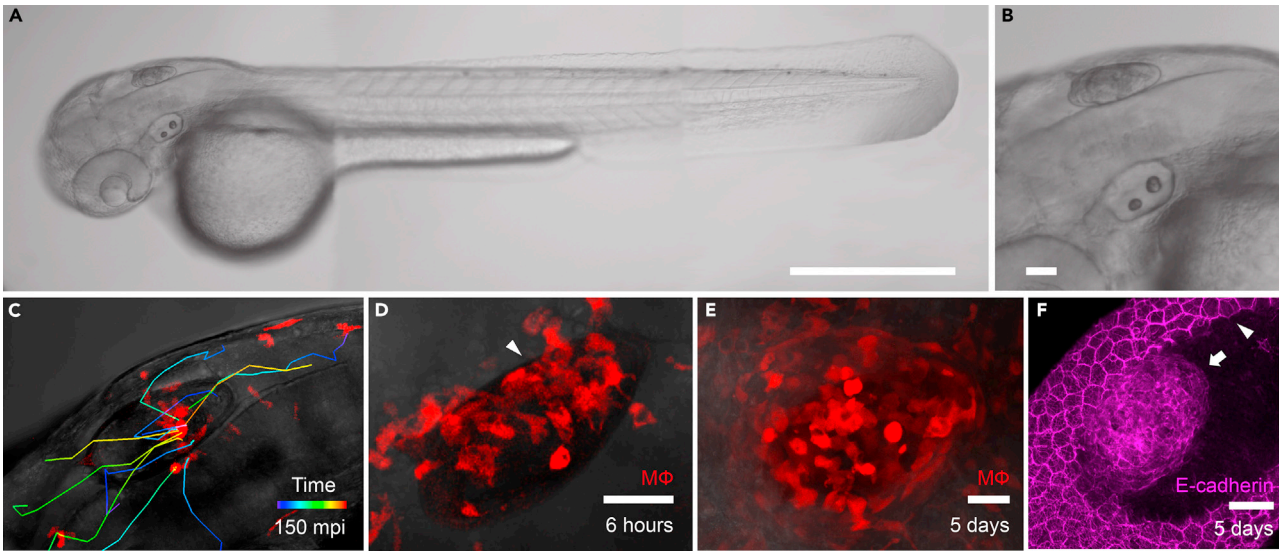

Figure 3. Immune responses following implantation of an immunogenic foreign body

(A and B) Zebrafish larva at 1 day post-implantation with a *S. mansoni* egg. (A) Scale bar, 500 μm. (B) Magnification of hindbrain ventricle with implanted egg. Scale bar, 25 μm.

(C and D) Early analysis of macrophage recruitment within 6 h post-implantation. (C) High temporal resolution tracking of macrophage migration from 60–150 min post-implantation (mpi) with colored tracks showing the route of migration. Color scale, longer wavelengths (red) represent later time points. Scale bar length, 50 μm. (D) Single time-point static imaging at 6 h post-implantation showing quantifiable recruitment of macrophages, and observable behaviors such as frustrated phagocytosis and aggregation (arrowhead). Scale bar, 25 μm.

(E and F) Granuloma formation at 5 days post-implantation. (E) Intravital image of granuloma, and then (F) immunostaining of E-cadherin showing epithelioid transformation of the granuloma (arrow). The surrounding structure is the epithelial lining of the hindbrain ventricle (arrowhead). (E and F) scale bars, 25 μm.

implantation. In the case of an immunogenic foreign body such as the schistosome egg, the initial immune response has been observed as macrophage recruitment. This seeding of macrophages may be observed by high temporal resolution microscopy to visualize and track the migration route of each individual macrophage over a 3-h period following implantation (Figure 3C).

Additionally, static imaging with high spatial resolution may be performed at a single time point for quantification of macrophage recruitment, and in the case of a strongly immunogenic foreign body, observation of behaviors such as frustrated phagocytosis and aggregation (Figure 3D). Frustrated phagocytosis is observed as the flattening of the macrophage on the surface of the foreign body, and indicates a high affinity between the phagocyte and foreign body (Figure 3D). Meanwhile, the macrophages aggregate and form an encasing structure, indicative of the foreign body having a stimulatory affect which induces the macrophages to produce their own chemotactic signals (Figure 3D).

After aggregating together, the macrophages may organize into a granuloma which encases the foreign material, a process which has been observed to occur within 3–5 days post-implantation (Figure 3E). To better encase the foreign material, these granuloma macrophages may become tightly interdigitated in a process known as epithelioid transformation, which can be confirmed by immunofluorescence detection of E-cadherin, a marker for epithelioid transformation (Figure 3F) (Cronan et al., 2016; Pagan and Ramakrishnan, 2018; Takaki et al., 2021a)

While this example is limited to the macrophage immune response to the schistosome egg, this protocol has been used with transgenic zebrafish expressing fluorescently labeled neutrophils to observe the neutrophil response, and has likewise been adapted to assess the immune response to a variety of implanted materials, include polymer beads (Takaki et al., 2021a).

Additional granuloma-inducing foreign materials which may be implanted include other species of schistosome eggs (e.g., *S. japonicum* and *S. haematobium*), pine pollen (which induces epithelioid granulomas), and silica. The immune response to specific antigens maybe be tested by implanting polymer beads which have been conjugated with various proteins and molecules of interest.

While the normally acellular hindbrain ventricle is ideal for assessing immune cell recruitment and response to foreign bodies following implantation, this protocol may be modified for implantation into other anatomical regions of the zebrafish. Implantation into the avascular region of the yolk sac may be used to assess angiogenesis (Rodríguez et al., 2012). Implantation into the brain may be used to directly observe the interaction of neurons with physical materials and devices in the study of brain machine interfaces.

LIMITATIONS

Although this protocol is written in step-by-step detail, implantation is a surgical procedure performed at the micrometer level, and as such, success is entirely dependent on the skill of the operator. In particular, a steady hand, fine motor skills, and hand-eye coordination are required traits of the operator.

When applying this protocol to new materials, size of the foreign material is an important consideration. Implanted materials must be large enough to induce foreign body reaction rather than phagocytosis, and large enough to observe cell-to-cell interactions on its surface, yet not so large that they exceed the capacity of the hindbrain ventricle. Based on these conditions, the minimum and maximum size of an object would be within the range of 10 μm–80 μm in diameter, with each extreme end of the range sufficient, but not ideal. The ideal size for an object is approximately 50 μm diameter. Another consideration is durability of the implanted material. Implanted materials must be durable enough to remain intact during the procedure. For example, self-made agarose microspheres below 1% were found to be too soft for implantation, a problem which could be solved by increasing the agarose percentage, or by cross-linking the agarose to improve durability.

Implantation is easiest within 30–48 h post-fertilization while larvae are soft. However, during this time the larvae possess innate-only immunity as conferred by macrophages and neutrophils. To study other immune cells, one possibility is to raise the implanted larvae until later timepoints when innate and adaptive immunity have fully developed. Alternatively, it may be possible to perform microimplantation in older larvae.

TROUBLESHOOTING

Problem 1

The entire larva was aspirated into the VAMP during usage (step 2a).

Potential solution

To avoid aspirating the larva during implantation, do not use excessive thumb pressure when grasping with the VAMP; gentle occlusion of the hole should result in gentle grasping of the larva. If difficulty persists, then the VAMP pressure may be too high, and may need adjustment. To reduce the aspiration pressure, small perforations and tissue paper maybe introduced to the tubing between the filter and the pump. If more precise adjustments are needed, a small airline control valve may be inserted between the filter and the pump.

Problem 2

There is no suction from the VAMP (step 2a).

Potential solution

The filter may have become wet. Replace the filter and ensure that water is not aspirated into the hosing during usage. Ensure that hosing is secure.

Problem 3

Incisions are difficult to make, and the hindbrain ventricle is difficult to target (step 2b).

Potential solution

As the larval zebrafish matures, its tegument becomes tougher, and the hindbrain ventricle becomes narrower and more elongated. Implantation should be performed within the optimal age-range, between 30–48 hpf, while the tegument is soft, and the hindbrain ventricle is a large target for implantation. If the problem persists, ensure that the CAIN is beveled to a fine point.

Problem 4

There is poor suction from the CAIN (step 2d).

Potential solution

The CAIN might be clogged or contain an air bubble. Attach a p1000 tip to the CAIN hosing and gently aspirate and expel the fish water from the tip to remove any clogs or air bubbles.

Problem 5

Survival is poor following microimplantation (step 2a).

Potential solution

The equipment may be contaminated. Disinfect the depression slide with 70% ethanol. Disinfect the tip of the VAMP by spraying or pipetting 500 μL into the thumb port, and then drain by touching the tip to a lab tissue. Allow to dry before usage. Ensure that equipment is clean and free of any pathogens which are not being studied.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kevin Takaki (kt422@cam.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100835.

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AUTHOR CONTRIBUTIONS

K.K.T. conceived and developed the tools and approaches used in this protocol. K.K.T. performed all experiments. K.K.T. wrote the manuscript.

DECLARATION OF INTERESTS

The author declares no competing interests.

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