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METHODS & TECHNIQUES

# High-throughput live-imaging of embryos in microwell arrays using a modular specimen mounting system

**Seth Donoughe[1,*], Chiyoung Kim[1] and Cassandra G. Extavour[1,2,*]**


## ABSTRACT

High-throughput live-imaging of embryos is an essential technique in developmental biology, but it is difficult and costly to mount and image embryos in consistent conditions. Here, we present OMMAwell, a simple, reusable device to easily mount dozens of embryos in arrays of agarose microwells with customizable dimensions and spacing. OMMAwell can be configured to mount specimens for upright or inverted microscopes, and includes a reservoir to hold live-imaging medium to maintain constant moisture and osmolarity of specimens during time-lapse imaging. All device components can be fabricated by cutting pieces from a sheet of acrylic using a laser cutter or by making them with a 3D printer. We demonstrate how to design a custom mold and use it to live-image dozens of embryos at a time. We include descriptions, schematics, and design files for 13 additional molds for nine animal species, including most major traditional laboratory models and a number of emerging model systems. Finally, we provide instructions for researchers to customize OMMAwell inserts for embryos or tissues not described herein.


**KEY WORDS: Embryogenesis, Microscopy, High-throughput, Time lapse, Image analysis, Quantitative imaging, Development**

## Introduction

Live-imaging embryos and small organisms in a repeatable, high-throughput manner is crucial for understanding the cellular dynamics that underlie the development of multicellular bodies (Farhadifar et al., 2015; Kuntz and Eisen, 2014). High-throughput imaging allows one to assess subtle phenotypes that can arise from functional genetics experiments, study standing variation within a population, and understand the role of noise in developmental processes. To that end, some research groups have turned to microfluidic devices (Chronis, 2010; Crane et al., 2010; Cornaglia et al., 2015; Wielhouwer et al., 2011). Such microfluidic apparatuses can be constructed to perform precise and complex experimental manipulations, but designing and fabricating these devices is a laborious process. For the purpose of imaging embryos, another option is to fabricate a custom mold that can be used to cast an agar or agarose microwell array. Molds can be milled from plastic (F. Kainz, Notch and FGF signalling in *Gryllus bimaculatus* and their role in segmentation, PhD thesis, Harvard University, 2009) or aluminum (Herrgen et al., 2009), or 3D-printed (Alessandri et al., 2017; Gregory and Veeman, 2013; Wittbrodt et al., 2014). Although these techniques are effective, each was designed to serve the specific needs of one particular species, and therefore it is not straightforward to adapt the existing tools to a new study species.

To address this outstanding need, we developed OMMAwell (Open Modular Mold for Agarose Microwells), an all-in-one device that allows the user to swap out any number of customized mold inserts. These inserts can be prototyped quickly and cheaply, requiring only a laser cutter or a 3D printer. These mold inserts lock into the device, which can be configured in several ways to mount specimens for any upright or inverted microscope that can accommodate a 35 mm petri dish. Using this tool, we can mount dozens of embryos at once in a microwell agarose array, keeping track of each embryo by its position in the array, and then efficiently image them. The modular mold inserts can be exchanged to alter the size, shape, orientation, and spacing of microwells. OMMAwell is therefore adaptable for different experimental designs or even diverse species.

As an example case, we demonstrate a workflow for making a custom mold insert for embryos of the cricket *Gryllus bimaculatus*. These cricket embryos can be imaged through their transparent eggshells. During previous efforts to live-image embryonic development within the eggs – using confocal and widefield microscopy – only a few embryos could be imaged at a time, and the mounting process was inconsistent and time-intensive (Donoughe and Extavour, 2016; Nakamura et al., 2010). Eggs were either manually glued to a coverslip one at a time (Nakamura et al., 2010) or placed in blocks of rubber polymer in which troughs had been hand-cut with a razor (Donoughe and Extavour, 2016). Mounting is similarly laborious for most animal laboratory models, which limits the sample size of experiments and reduces reproducibility. However, we show that with OMMAwell, it is straightforward to mount dozens of embryos in a manner that is suitable for 2D or 3D long-term time-lapse recordings.

In the Supplemental Information, we include detailed instructions for assembling the OMMAwell mounting device, and suggestions for modifying the device to suit the particular requirements of any desired model system. We have also designed and beta-tested mold inserts for embryos of eight additional species, including zebrafish, fruit fly, frog, annelid worm, amphipod crustacean, red flour beetle, and three-banded panther worm, as well as mouse neurospheres. Descriptions, schematics, and design files for all of these mold inserts are provided.

## Results and Discussion

To design the first iteration of a cricket embryo mold, we collected and measured dimensions of freshly laid eggs (Fig. 1A). The eggs are roughly ellipsoidal in shape, 2500–3200 µm in length, and 475–650 µm in width (Fig. 1B,C). We designed the mold insert to have

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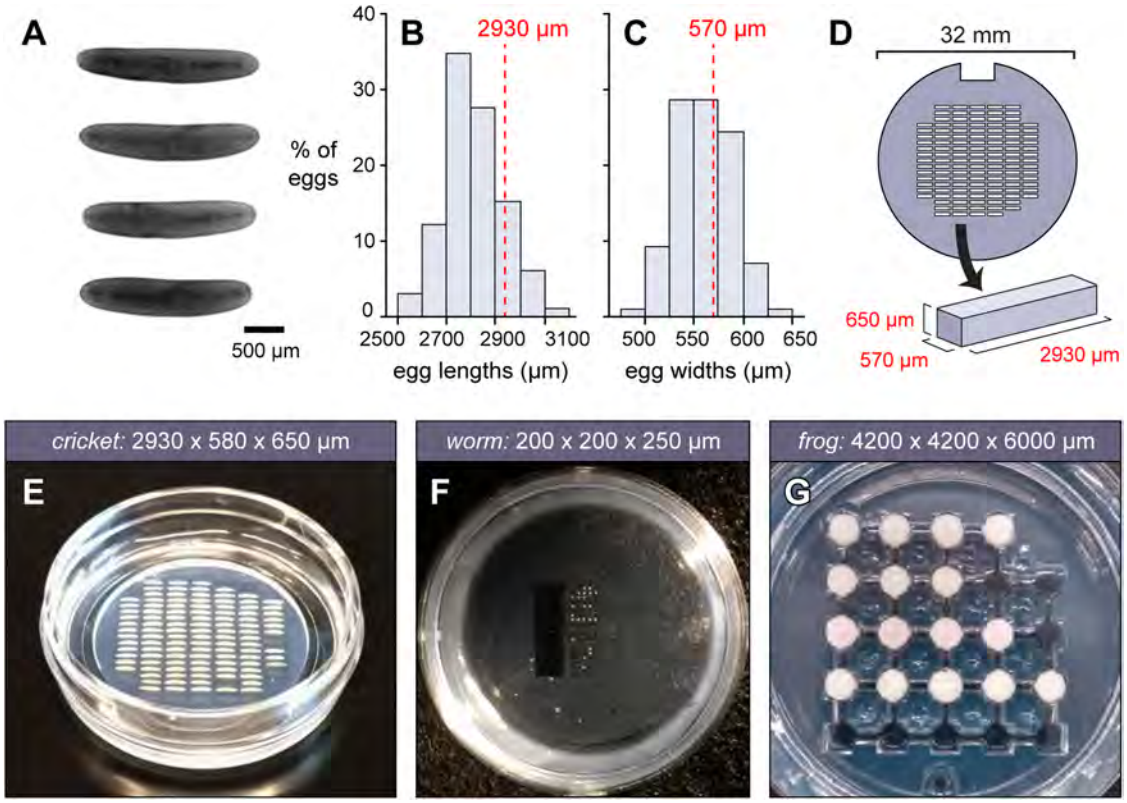


**Fig. 1. Designing microwells to hold cricket eggs.** (A) Freshly laid cricket eggs were measured and their (B) lengths and (C) widths plotted ($n$=98; scale bar: 500 µm). Based on the size distribution, we chose dimensions of 2930×570×650 µm (embryo length×width×height), which were values such that approximately 75% of eggs would fit into the troughs. In practice, because the wells are made of agarose, more than 95% of eggs fit into these wells. (D) Raised posts of those dimensions were formed by engraving an acrylic insert. 120 such posts were arranged in a grid. (E) Agarose microwells made using this insert, loaded with cricket eggs. (F,G) Example molds for an annelid worm and coqui frog, with microwell dimensions listed. Photos in F and G by Elaine Seaver (The Whitney Laboratory for Marine Bioscience) and Mara Laslo (Harvard University) respectively.

rectangular posts 2930 µm long by 570 µm wide, each of which will create an agarose microwell able to snugly accommodate the majority of eggs (Fig. 1D).

The 'mold insert' is the only piece of the OMMAwell that must be tailored to create wells of appropriate dimensions for one's samples of interest. To make the cricket mold insert, the inverse of our desired pattern was laser-engraved into acrylic to a depth of 650 µm. This is deep enough to contain the embryo, but close enough to the surface to be imaged within the working distance of 5× and 10× microscope objectives. The microwells were arranged into a truncated grid pattern that fit within a 26 mm circle, so that all microwells could be viewed through the circular 27 mm in diameter coverslip (surface area 531 $mm^2$) of a 35 mm glass-bottom petri dish (Fig. 1D,E; see the Supplemental Information). Given the dimensions of these particular embryos, we were able to fit 120 wells into the grid. For embryos of different dimensions, more or fewer wells may be able to fit into the coverslip field (e.g. 24 wells for the coquí frog *Eleutherodactylus coqui*; 294 wells for the fruit fly *Drosophila melanogaster*; see the Supplemental Information). One post was omitted in one corner, to make it possible to unambiguously orient the dish.

The resulting microwells for cricket embryos are shown in Fig. 1E, alongside example microwells for two additional species, the three-banded panther worm *Hofstenia miamia* (Fig. 1F) and *E. coqui* (Fig. 1G). Note that in the latter two cases, the mold microwells were not simple rectangles, but instead had more complex shapes. Since the mold inserts are designed in 2D using a simple drawing program (see Supplemental Materials S1), it is easy for a user without prior experience to design and iterate a complex custom mold. Details for all 14 user-tested mold inserts are in Supplemental Materials S1.

With the mold insert ready, we cut and assembled the non-customized OMMAwell components. Detailed assembly instructions with photo guides are in Supplemental Materials S2; the design file for each component is included in Drawing Exchange Format (DXF) and Portable Document Format (PDF) in Supplemental Data. These files can be opened and edited by many design or drawing software packages, including AutoCAD, FreeCAD, Solidworks, SketchUp Pro, Adobe Illustrator, and CorelDRAW. All of the pieces can be made from a single sheet of 6 mm thick acrylic sheet on a laser cutter, which is how we fabricated them for testing. Another option is to 3D print the components by using the included design files as the basis for a 3D model of each piece. If the user does not have access to a laser cutter or 3D printer, pieces can also be fabricated by a variety of online providers.

For a single mold insert, there are three possible OMMAwell configurations, each of which is useful for different purposes (Figs 2 and 3). Below we discuss the use of each configuration separately.

Configuration 1: Top loaded microwells for injecting or imaging with an upright microscope (Fig. 2A; see legend for step-by-step usage instructions). In this arrangement, the user can adjust the height of the mold insert, which is then lowered into molten 1.5%

Biology Open (2018) 7, bio031260. doi:10.1242/bio.031260

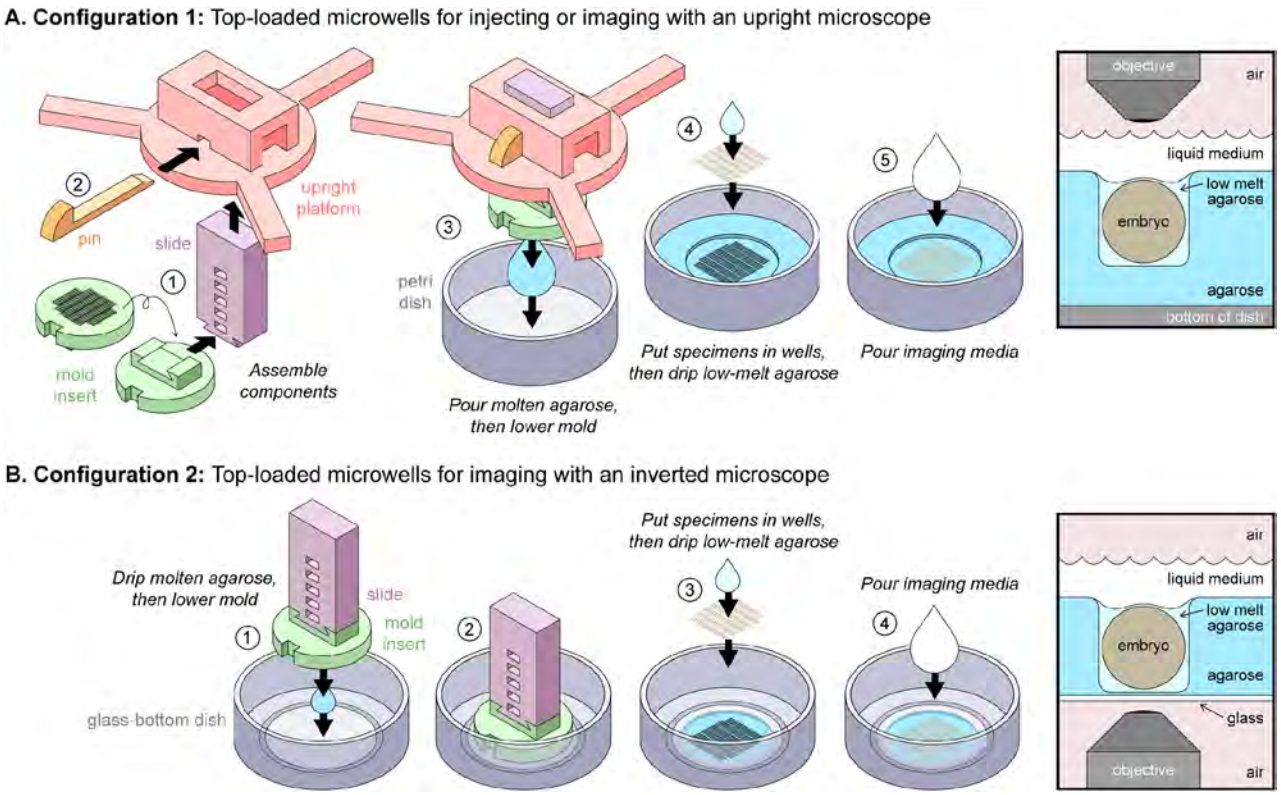


**Fig. 2. OMMAwell configurations for top-loaded microwells.** (A) Configuration 1: Top loaded microwells for injecting or imaging with an upright microscope. (1) The mold insert (green) is inverted and connected to the slide (purple), which is placed into the upright platform (pink). (2) After the desired height is chosen, the pin (orange) is inserted. (3) Molten agarose is poured into a plastic petri dish and the mold assembly is lowered into it. (4) After the agarose sets, the mold insert is removed and eggs are placed into the wells, either individually with forceps or many at once by transferring the eggs in water with a cut plastic pipette. Excess water is removed by pipet and then wicked away with piece of lint-free lens paper. Then, 40–100 µl of molten low-melt agarose, kept at 42°C, is added to the wells to hold the eggs in place. Embryo positions are adjusted with plastic forceps. (5) When the low-melt agarose sets, the live-imaging medium is added to the dish. Right: Schematic of embryo in Configuration 1. (B) Configuration 2: Top loaded microwells for imaging with an inverted microscope. (1) 700 µl of agarose is pipetted into the middle of the glass-bottom dish. The insert and slide are lowered onto it, taking care not to trap bubbles. (2) Agarose sets, and then the insert and slide are gently removed. (3) Embryos are loaded into microwells, as described above. (4) Live-imaging medium is added. Right: Schematic of embryo in Configuration 2.

(w/v) agarose. Once the agarose has cooled and set, the mold is removed, leaving microwells in which to place the samples. Optionally, a small quantity of 0.7% (w/v) low-melt agarose (40–100 µl) can then be added to hold samples in the wells. When they are fixed in place, the live-imaging medium is added. This configuration is well-suited for dipping microscope objectives.

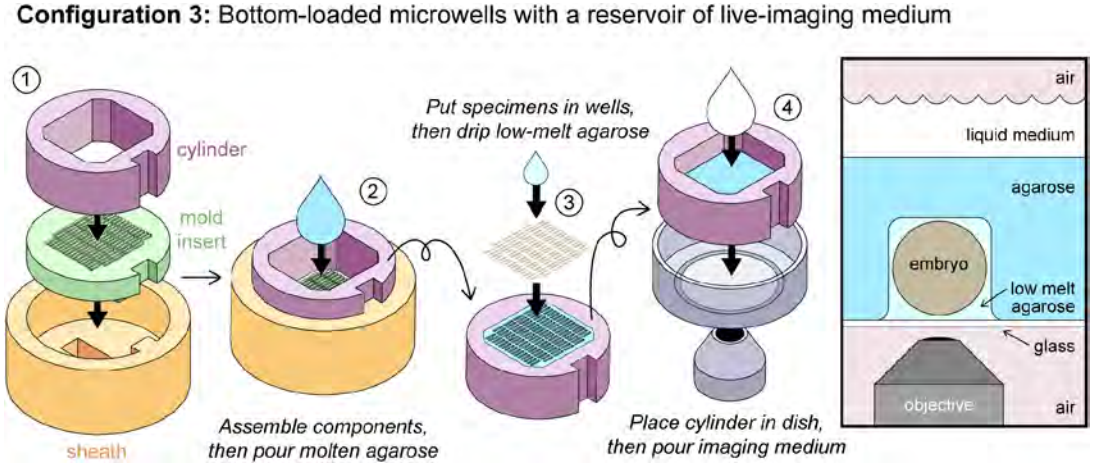


**Fig. 3. OMMAwell configurations for bottom-loaded microwells.** Configuration 3: Bottom loaded microwells with a reservoir of live-imaging medium. (1) The insert (green) and cylinder (purple) are placed into the sheath (orange). (2) Molten agarose is poured into the cylinder to the desired depth. (3) Once the agarose has set, the cylinder and the agarose block are removed from the sheath and insert. The cylinder is flipped over, and the exposed microwells are loaded with embryos, as described in Fig. 2. (4) The cylinder and agarose block are lowered into the glass-bottom dish, and live-imaging medium is added in the cylinder. Right: Schematic of embryo in Configuration 3.

Biology Open (2018) 7, bio031260. doi:10.1242/bio.031260

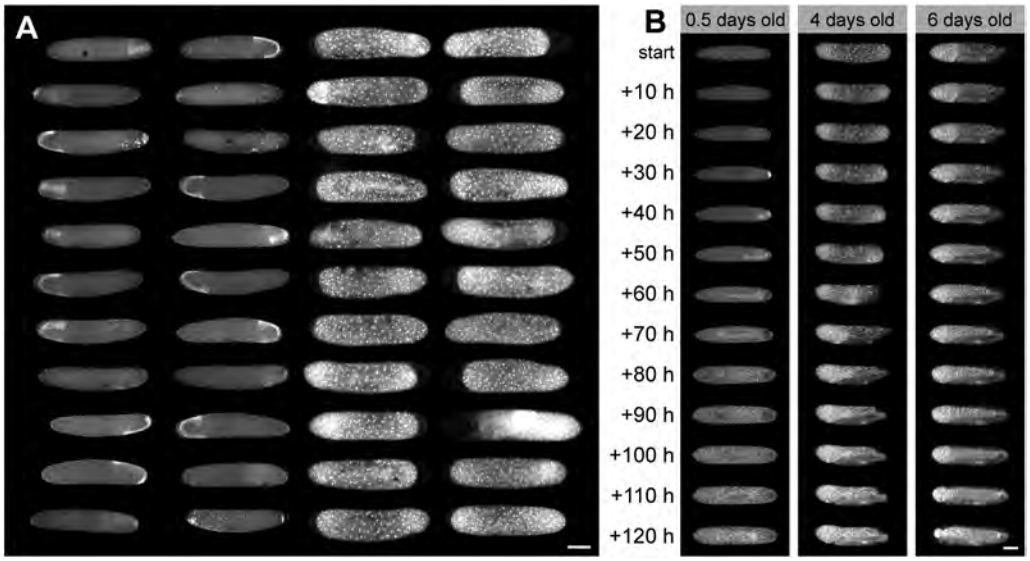


**Fig. 4. Arrays of live-imaged embryos within the OMMAwell mold.** (A) A single timepoint from a time-lapse of an array of nuclear-marked transgenic cricket embryos in microwells. The two leftmost columns show germ band stage embryos that are beginning the physical re-orientation within the egg called anatrepsis. The two rightmost columns show a later stage when embryos are fully immersed in the yolk below the extraembryonic membrane called the serosa. (B) Time series of cricket embryos starting at different ages. The ambient temperature is ∼24°C, so development is slower than that reported by Donoughe and Extavour (2016). Scale bars: 500 µm.

It is also useful for holding samples that will be injected, such as with double-stranded RNA, small molecule activators or inhibitors, or recombinant protein (Donoughe et al., 2014). In this configuration, cricket embryos will successfully complete embryogenesis (∼12 days), so long as the medium level is maintained. A drawback of this configuration is that if the working distance of the microscope objective is too short, a lid cannot be added to prevent evaporation. Configurations 2 (Fig. 2B) and 3 (Fig. 3) do not have this drawback.

Configuration 2: Top loaded microwells for imaging with an inverted microscope (Fig. 2B; see legend for step-by-step usage instructions). This is similar to Configuration 1, but the mold insert is placed flat on molten 1.5% (w/v) agarose in a glass-bottom dish, producing microwells in a thin agarose film. The samples are loaded into the wells, fixed in place with 0.7% (w/v) low-melt agarose as above, the dish is covered with its lid, and then imaged from below on an inverted microscope. Because the lid remains on the dish and reduces evaporation, this configuration is the best one for long-term live imaging. As with Configuration 1, but without the need to maintain the medium level manually, cricket embryos mounted in Configuration 2 will develop normally for 12 straight days, completing embryogenesis at rates comparable to unmounted embryos (92–100% hatching). A minor difficulty of this configuration may be that removing the mold insert and slide (Fig. 2B, Step 2) without disrupting the thin agarose film can be a delicate procedure for some insert designs. To ameliorate this problem, we recommend the use of 2% (w/v) agarose to make the microwells. When it has set, pull up the mold insert with the agarose still adhered to it. Then, using plastic forceps, peel the agarose from the insert, return it to the glass-bottom dish, and 'glue' it in place with ∼100 µl of 0.7% low-melt agarose. It can take first-time users some practice to become effective in peeling the agarose from the insert, but once it has been peeled, we do not notice any non-uniformities in the wells. Since this configuration relies more strongly than the others on manual manipulation, this technique has more opportunities for variance than the others. In our hands, however, it is a trade-off that can be worth making for some experiments.

Configuration 3: Bottom-loaded microwells with a reservoir of live-imaging medium (Fig. 3; see legend for step-by-step usage instructions). This configuration is recommended for cases where making the agarose film in Configuration 2 is troublesome for a particularly complex mold insert, or if it is necessary to use a larger volume of imaging medium than can be poured into the glass-bottom dish. The main advantage over Configuration 2 is that the mounting process is extremely robust. The downside is that the samples are separated from the imaging medium by a much thicker layer of agarose, which means that gas exchange is reduced. In our hands, cricket embryos mounted in this fashion will typically develop normally for only 6–12 h and then arrest. If the embryos are subsequently removed from their microwells and immersed in water, development continues normally. This configuration also offers a larger reservoir that can be filled and capped with a lid; its volume can be increased further by adding more layers to the 'cylinder' in Step 5 of Supplemental Materials S2.

We have used each of these three configurations (Figs 2 and 3) to live-image more than 100 embryos simultaneously. In some species, embryonic development may be particularly sensitive to oxygen supply. If this is a concern, Configuration 2 is most suitable, as it minimizes the amount of agarose around the embryos. For our work with crickets, we can oxygenate embryos by manually bubbling and stirring the imaging media, although this is not required for healthy development. An automated approach for oxygenation would require additional tool development that we have not explored. For species with smaller embryos, the maximum sample size is even larger, and it is up to the user to determine the desired density and number of wells. If the array of wells is larger than the microscope's field of view, the user can either manually move the stage or use a motorized stage to move the array in the X-Y plane so that all the samples can be imaged. Because each well has a unique identifier, even with a manual approach, large numbers of individual embryos can be followed and uniquely identified over time-lapse periods. As an example, we show a single time point from a time-lapse of 44 nuclear-marked transgenic cricket embryos (Fig. 4A). We used a motorized stage to capture tiled micrographs of the full set of eggs once every 5 min. The recording continued for 5 days with no signs of phototoxicity or developmental defects. The specimens were then returned to the incubator, and 41 of the 44 embryos hatched, survivorship that is comparable to embryos that were not mounted or imaged (this ranges from 90–100% across trials). We do not observe developmental delays in imaged embryos. In the case of crickets, we routinely transfer embryos from the agarose wells to another dish, mid-development, with no loss in embryo integrity. We have not systematically tested survivorship

following this procedure with other species. For species with fragile embryos, the question of whether or not such a transfer procedure might be possible without unacceptably compromising survivorship would have to be determined empirically.

Researchers can easily design and fabricate their own mold inserts to generate wells of specified shapes, dimensions, and spacing. Patterns can be designed *de novo*, or altered from the insert files included with this article (Supplemental Data). If the user fabricates a piece from acrylic using a laser cutter, the design can be simply made as a 2D form, like those described in Supplemental Materials S1. If the user prefers to a use a 3D printer, the included design files can be used as the basis for creating a new 3D design file. A brief comparison of the advantages and disadvantages of each mode of fabrication is given in Supplemental Materials S3.

## Materials and Methods

Design and assembly details are given in Supplemental Materials S1 and S2. When making the microwells, agarose (Bio-Rad #1613101) was dissolved at 1.5% weight/volume (w/v) in distilled water (or 2% for firmer molds). Then, eggs were held in microwells with low-melt agarose (Bio-Rad #1613112) at 0.7% (w/v) in distilled water. Tap water was used as a live-culturing solution for wild-type cricket eggs, but the user could also pour molds with agarose dissolved in a live-imaging buffer that is appropriate for their samples.

*G. bimaculatus* wild-type strain was originally reared in Yamagata prefecture, Japan. Wild-type eggs were imaged with transmitted white light on a Zeiss Lumar dissection microscope. For fluorescent imaging, recordings were taken of eggs from a transgenic line in which the cricket actin promoter drives expression of the cricket Histone-2B protein fused to Enhanced Green Fluorescent Protein (H2B-EGFP) (Nakamura et al., 2010). The 5× objective on a Zeiss Celldiscoverer 7 was used for imaging. We have also successfully imaged OMMAwell-mounted embryos with 10× and 20× objectives, but we have not systematically tested all mounting options on these and other higher magnification objectives. In each case, success or failure will depend on the working distance of the objective, the size of the specimens, and which of the three OMMAwell configurations is being used. The array of microwells was tiled with the motorized stage under the control of Zen software (Zeiss, Oberkochen, Germany). A z-stack was captured at each position, then later combined using Zen's 'Extended Depth of Focus' (mode='Contrast'). Figures were prepared using Illustrator (Adobe).

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: S.D.; Methodology: S.D., C.K.; Validation: S.D., C.K.; Investigation: S.D.; Writing - original draft: S.D.; Writing - review & editing: C.G.E., S.D.; Supervision: C.G.E., S.D.; Funding acquisition: C.G.E.

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#### Supplementary information

Supplementary information available online at
http://bio.biologists.org/lookup/doi/10.1242/bio.031260.supplemental

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# Supplemental materials S1: Design files for user-tested mold inserts

# Design files for user-tested mold inserts

This document contains descriptions and schematics for 14 mold inserts that were designed to be used for a range of study organisms, including zebrafish, fruit fly, mouse, frog, annelid worm, amphipod, red flour beetle, acoel, and cricket. Each insert is compatible with the adjustable-height mounting tripod described elsewhere in this paper.

Components were designed using FreeCAD and Adobe Illustrator. Each mold component was made by cutting it from a sheet of 6.35 mm optically clear extruded acrylic (McMaster-Carr #8560k355). A laser cutter (Universal PLS 6.75) in engraving mode was used to generate mold inserts by removing acrylic in a pattern, leaving a grid of raised posts. When pieces needed to be combined, they were fused together in pairs using acrylic welding solvent (Weld-on 3 Assembly Adhesive, SciGrip #10799) (see Supplemental Materials S3 for details). Most of these mold inserts were designed to fit within a 35 mm diameter plastic petri dish with a 27 mm diameter glass coverslip bottom (VWR #89428-990). Many microscopes need a stage insert in order to stably hold a petri dish (e.g. Zeiss #432311-9901-000). In Configuration 1, the device can be used on a range of dish sizes; we tested it on 6 cm (VWR #25384-092) and 10 cm (VWR #25384-342) diameter plastic petri dishes.

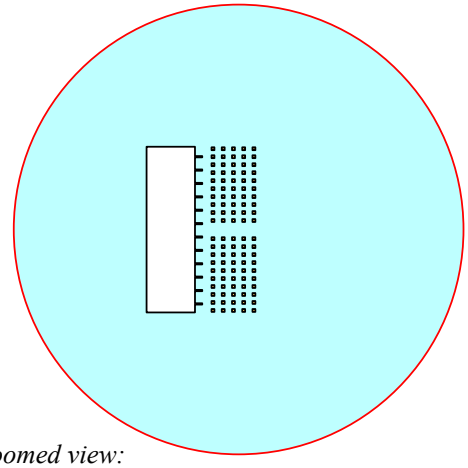
To use these inserts with the OMMAwell device, simply attach slots to the back of the insert, following the procedure in step #7 of the assembly instructions in Supplemental Materials S3. Details for each insert are included below. Each schematic has been magnified for presentation and the key dimensions are given with each insert. For complete dimensions, please see the design files in Supplemental Materials S2. These are included in two formats: PDF and DXF. PDF files are readily viewed and edited by many common software packages. DXF is a file type that can be opened in a wide variety of drafting and vector graphics software packages, including AutoCAD, FreeCAD, Solidworks, SketchUp Pro, Adobe Illustrator, and CorelDRAW. Unlike PDF, DXF files contain precise length information. We hope readers will use these as a starting point to make their own inserts; if so we would be happy to hear about it. Each of these mold inserts can be made from 6 mm-thick acrylic sheet using a laser cutter. Alternatively, if a user prefers to fabricate them with a 3D printer, the user can adapt the included design files to make a 3D model of each component. The depth dimensions of each part is listed below. See Supplemental Materials S4 for a brief comparison of the two approaches.

**Identifying wells:** For some molds, there are symbols or numbers to identify columns or rows on the agarose wells. These provide the "coordinate address" of each well. In other molds, the arrangement of the molds themselves – that is, an arrangement without rotational symmetry – is sufficient information to uniquely identify each well.

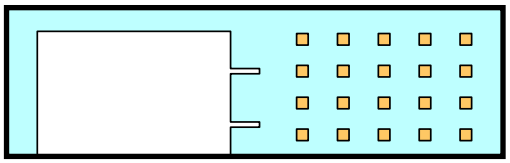
**Note:** At the end of this document is a section that discusses considerations for using wells to hold specimens for injection.

## *Capitella teleta,* 100-well

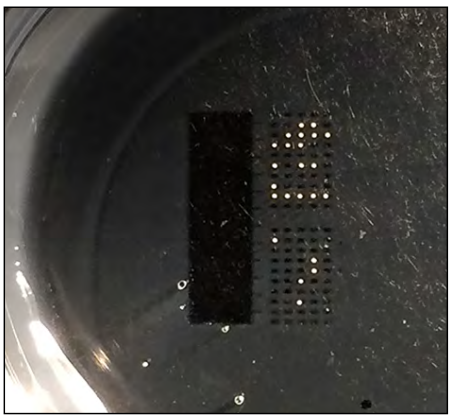
`Insert_Capitella_Injection_200x200um_100n`

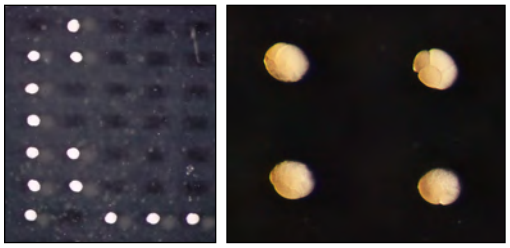


*Zoomed view:*



**Description:** This mold layout was designed in collaboration Dr. Mark Martindale (University of Florida), Dr. Elaine Seaver (University of Florida), and Dr. Mansi Srivastava (Harvard University), for injecting the spherical eggs of marine invertebrates and acoel worms. Eggs are placed into the square wells and then injected with a pulled glass needle entering from the right. When an egg gets stuck on the needle, the user can move the needle tip into the large well on the left, and then slide the needle up and out through one of the thin troughs, freeing the egg from the needle. This mold been tested with eggs from the marine annelid worm *Capitella teleta*, but by adjusting the dimensions of the square microwells, this mold insert can easily by adapted to a wide range of species. To make this mold, cut on the **red** line, etch the **cyan** region to a depth of ~800 µm, and etch the **orange** regions to a depth of ~550 µm.



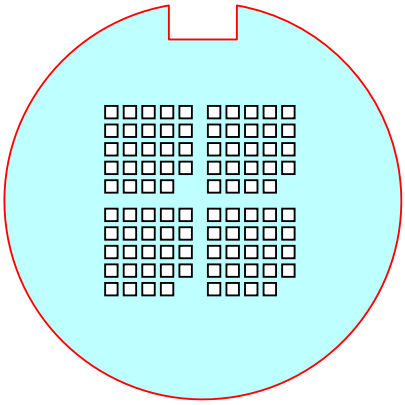


**Dimensions:** This insert generates agarose microwells that are 200 µm square, with a depth of 250 µm. The large well on the left has a depth of 800 µm. The outer margin of the insert is a 31 mm diameter circle to fit in a 35 x 10 mm plastic dish (Bio-One #627160) for injection.

Photos by Elaine Seaver (University of Florida)

## *Danio rerio,* chorions intact, 96-well

`Insert_Danio_1100x1100um_96n`

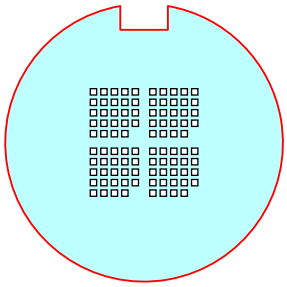


**Description:** This mold was designed for injecting and live-imaging zebrafish embryos with their chorion intact. To make this mold, cut on the **red** line, then etch the **cyan** region to a depth of ~1300 µm.

**Dimensions:** Each microwell is 1100 µm square, with a depth of 1100 µm. The outer margin of the insert is a circle with radius 35 mm to fit snugly into a glass-bottom dish for live-imaging with an inverted microscope and into the mold sheath (see Supplemental Materials 3). The wells are placed so that the embryos all land within the 27 mm diameter of the glass coverslip bottom (VWR #89428-990). The same is true for all mold inserts described in this document that have a rectangular notch.

## *Danio rerio,* chorions removed, 96-well

`Insert_Danio_800x800um_96n`



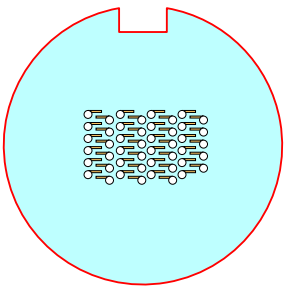
Photos by Megan Norris (Harvard University)

**Description:** This mold is used for live-imaging zebrafish embryos whose chorions have been removed. To make this mold, cut on the **red** line, then etch the **cyan** region to a depth of ~800 µm.

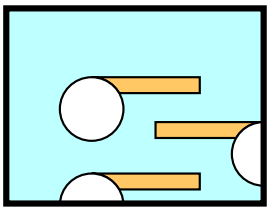
**Dimensions:** Each microwell is 800 µm square, with a depth of 1000 µm. The outer margin of the insert is a circle with radius 35 mm. It is compatible with the glass-bottom dishes and the mold sheath, as described above.

## *Danio rerio,* embryos with growing tails, 47-well

`Insert_Danio_Lateral_Wells_47n`



*Zoomed view:*

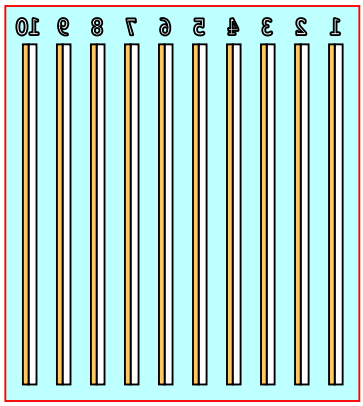


**Description:** During embryogenesis, the growing tail of zebrafish embryos makes it difficult to keep them in place during long term live-imaging. To address this problem, this mold generates circular wells for the spherical yolks to settle into, and adjacent troughs for the elongating tails to enter. To make this mold, cut on the **red** line, etch the **cyan** region to a depth of ~500 µm, and etch the **orange** regions to a depth of ~250 µm. *Note:* at these depths, the embryos's midline is approximately flush with the upper surface of the agarose, meaning that the larva protrudes upwards, making this mold best suited for imaging on an upright microscope, but if the etchings were 2-fold deeper, it would be suitable for inverted microscopy as well.

**Dimensions:** The circular portion of each microwell is 1 mm in diameter, with a depth of 0.5 mm. The tail trough is 1.7 mm long, 0.25 mm wide, and 0.25 mm deep. This mold is compatible with the mold sheath and the aforementioned glass-bottom dishes.

## *Danio rerio,* injection troughs

`Insert_Danio_Injection_Troughs_10n`

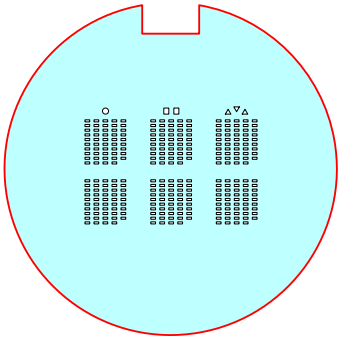



**Description:** This mold makes troughs that can hold many zebrafish eggs for injection. Each trough has a stepped notch removed from one side so that the needle can enter the eggs without getting stuck in the agarose. To make this mold, cut on the **red** line, etch the **cyan** region to a depth of ~700 µm, and etch the **orange** regions to a depth of ~350 µm.

**Dimensions:** Each trough is 30 mm long and 0.7 mm deep at the bottom. A trough is 0.7 mm wide at the bottom, and 1.2 mm wide at the top. The outer margin of the insert is a rectangle 31.2 by 34.8 mm. This insert will not fit in the mold sheath, but once slots are attached to the back, it can be used with the adjustable-height tripod.

## *Drosophila melanogaster,* 294-well

`Insert_Drosophila_220x500um_294n`

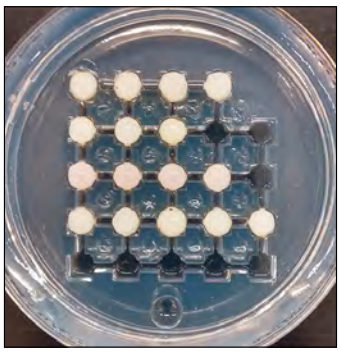


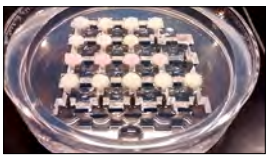
**Description:** This mold was designed for live-imaging arrays of fruit fly embryos. The etched shapes make it easier to navigate the array of microwells while using a microscope. To make this mold, cut on the **red** line, then etch the **cyan** region to a depth of ~240 µm.

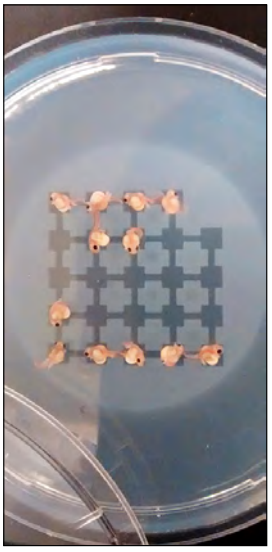
**Dimensions:** Each microwell is 500 µm long, 220 µm wide, and 240 µm deep. The outer margin of the insert is a circle with radius 35 mm. It is compatible with glass-bottom dishes and the mold sheath.

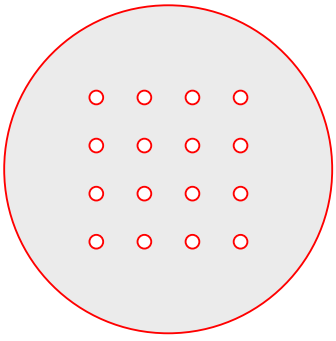
## *Eleutherodactylus coqui,* 24-well

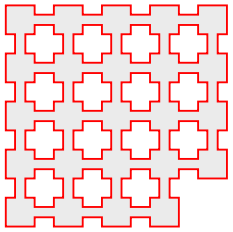
`Insert_Eleutherodactylus_4200x4200um_24n`











**Description:** This mold insert was designed to produce wells for injecting and live-imaging the embryos and larvae of the coquí frog. The channels in the agarose admit the tails of the larval frogs so that they can more easily be oriented by the user for microscopy. To make this mold, cut on **red** lines. Then use acrylic solvent (see Supplemental Materials S3 for details) or cyanoacrylate glue to attach the two pieces flat together, lining up the circular holes on the circular piece with the plus-sign-shaped holes on the rectangular piece. The holes allow air to enter and release the vacuum when removing the mold from the agarose.

**Dimensions:** Each well is 4.2 mm square; the channels connecting the wells are 1.5 mm wide. The array of wells is a square 32.2 mm to a side. The circular baseplate has a diameter of 47.8 mm. By varying the amount of agarose poured the user can adjust the depth of the wells.

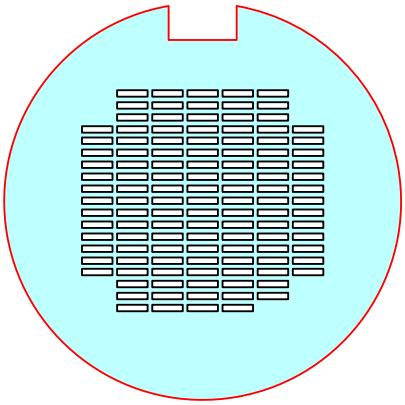
Photos by Mara Laslo (Harvard University)

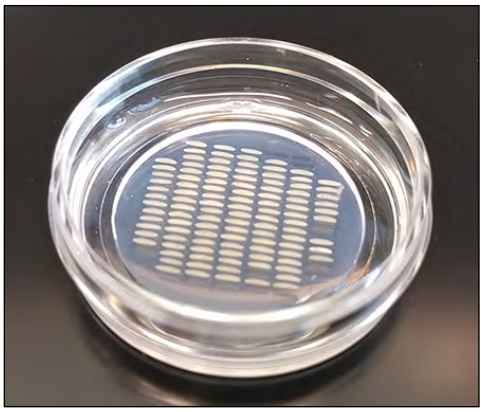
## *Gryllus bimaculatus,* 120-well

`Insert_Gryllus_570x2930um_120n`

**Description:** This mold insert was designed to fit the maximum number of cricket embryos on a single glass-bottom dish. Cut on **red** lines, and etch the **cyan** region to a depth of ~650µm.

**Dimensions:** Each microwell is 2930µm long, 570µm wide, and 650 µm deep. The outer margin of the insert is a circle with radius 35 mm. It is compatible with glass-bottom dishes and the mold sheath.



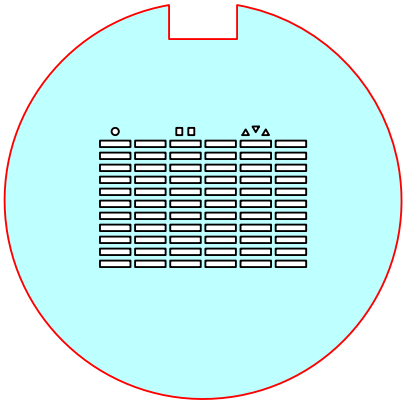


## *Gryllus bimaculatus,* 66-well

`Insert_Gryllus_570x2930um_66n`

**Description:** This is similar to the previous mold insert, but with fewer wells and etched shapes to aid in the orientation on the microscope. Wells are arranged into a rectangle for time-efficient automated tiling. Cut on **red** lines, and etch the **cyan** region to a depth of ~650µm.

**Dimensions:** Same dimensions as previous insert.



## *Gryllus bimaculatus,* 300-well

`Insert_Gryllus_Injection_570x2930um_300n`

**Description:** This mold insert is used for injecting large numbers of cricket eggs at once. Wells are arranged in groups of ten and labeled with an etched number. Cut on **red** lines, and etch the **cyan** region to a depth of ~750µm.

**Dimensions:** Each microwell is 2930µm long, 570µm wide, and 750 µm deep. Depending on the user's microinjection apparatus it may be helpful to adjust the depth of the wells.

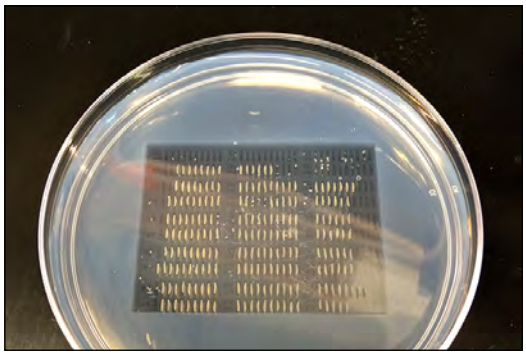
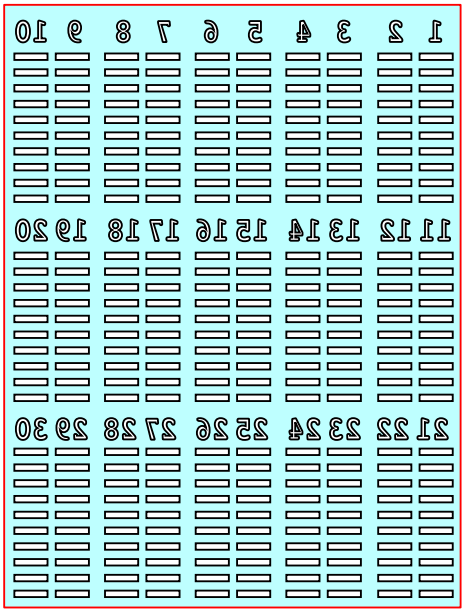
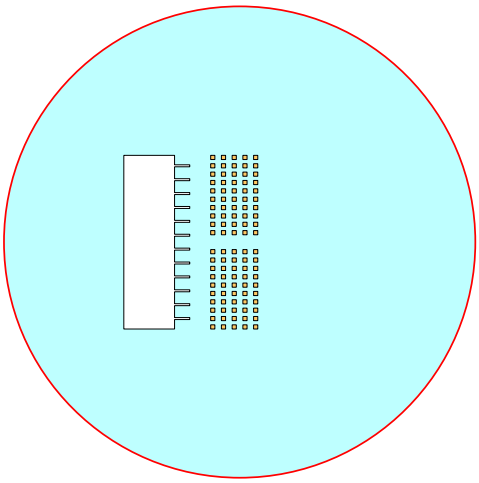


Photo by Taro Nakamura (Harvard University)



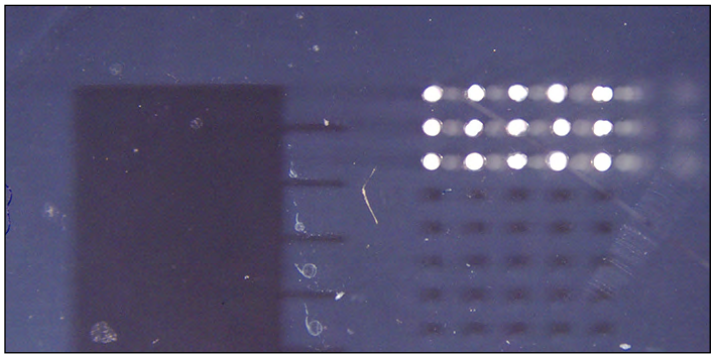
## *Hofstenia miamia,* 100-well

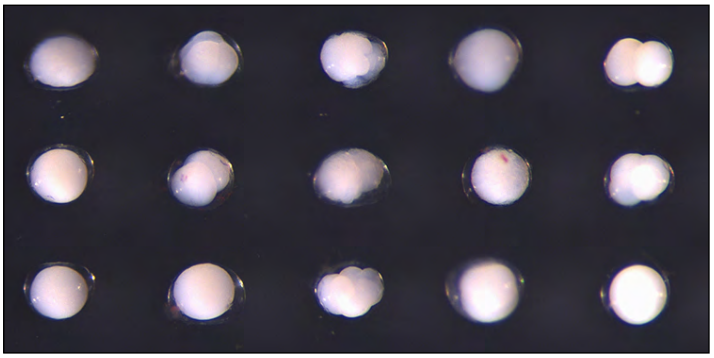
`Insert_Hofstenia_Injection_275x275um_100n`



**Description:** This mold insert is quite similar to the *Capitella teleta* insert described at the beginning of this document; the only difference is that the wells are larger to fit the eggs of the acoel worm *Hofstenia miamia*. To make this mold, cut on the **red** line, etch the **cyan** region to a depth of ~800 µm, and etch the **orange** regions to a depth of ~500 µm.

**Dimensions:** The insert generates agarose microwells that are 275 µm square, with a depth of 300 µm. The large well on the left has a depth of 800 µm. The outer margin of the insert is a circle with radius 31 mm to fit in a 35 x 10 mm plastic dish (Bio-One #627160) for injection.

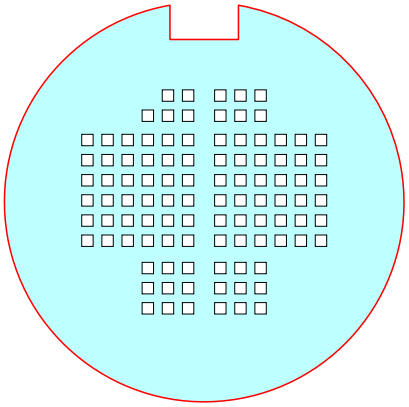




Photos by Andrew Gehrke (Harvard University)

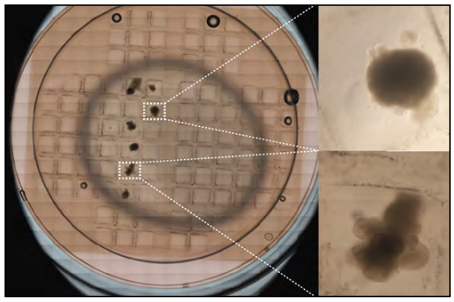
## *Mus musculus,* neurospheres, 101-well

`Insert_Mus_Neurospheres_1000x1000um_101n`



**Description:** Neurospheres are clusters of cultured cells derived from neural stem cells. This mold insert was designed to make wells to hold neurospheres for imaging. Cut on **red** lines, and etch the **cyan** region to a depth of ~1000µm.

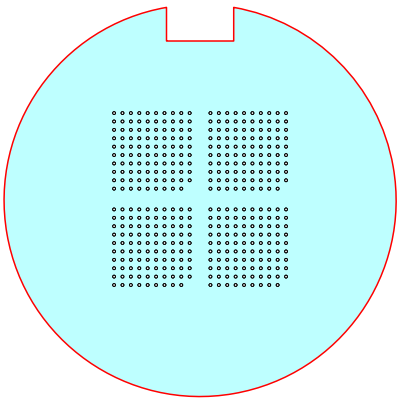
**Dimensions:** Each microwell is a cube 1 mm to a side. The insert is compatible with glass-bottom dishes and the mold sheath.



Photos by Richard Smith (Harvard Medical School)

## *Parhyale hawaiensis,* 396-well

`Insert_Parhyale_round250um_396n`



**Description:** *Parhyale hawaiensis* is a marine amphipod crustacean, an emerging model system for the study of regeneration, germ cell specification, and limb differentiation. This mold insert was designed to make wells to hold its spherical embryos for injection and live-imaging. Cut on **red** lines, and etch the **cyan** region to a depth of ~300 µm.

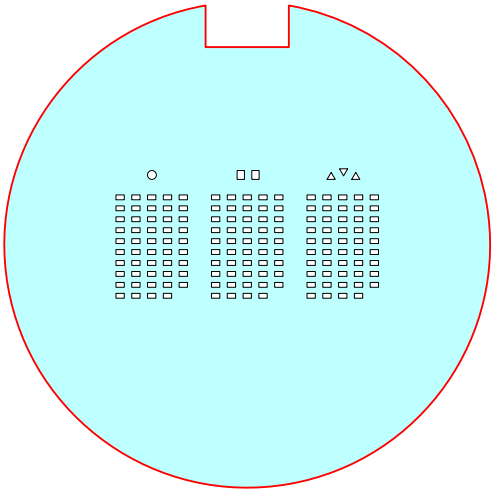
**Dimensions:** Each microwell is 250 µm long, 250 µm wide, and 300 µm deep. The insert is compatible with glass-bottom dishes and the mold sheath.

## *Tribolium castaneum,* 147-well

`Insert_Tribolium_350x600um_147n`

**Description:** The red flour beetle is a pest of stored grain products and an emerging model system for a range of animal developmental processes. This mold was designed to produce wells that fit the embryos for timelapse live-imaging. Cut on **red** lines, and etch the **cyan** region to a depth of ~375 µm.

**Dimensions:** Each microwell is 600 µm long, 350 µm wide, and 375 µm deep. The insert is compatible with glass-bottom dishes and the mold sheath.



# Considerations for using wells to hold specimens for injection

Several of the molds listed here have been used for successful injections, including the molds for zebrafish, cricket, coqui frog, three-banded panther worm, and annelid worm. When users test injection protocols for additional organisms, these molds and the usage described above my be helpful, but every species will have idiosyncratic challenges.

For instance, some embryos increase in size and/or become motile during development, which might necessitate a transfer from the injection wells to another vessel for subsequent development. Such transfers work effectively in crickets, amphipods, and coqui frogs. We have not tested mid-embryogenesis transfers for other species. Another potential concern is that eggs of some species might rotate under the injection needle. One possible solution for this is to adjust the shape of the well so that the egg can be jammed into a corner during injection.

Some users have also found it helpful to adjust the osmolarity of the acqueous medium in order to increase (or decrease) the swelling of specimens in the wells during injection.

# Supplemental materials S2: Guide for assembling OMMAwell

## Guide for assembling OMMAwell

### Materials

- Extruded acrylic, 6.00 to 6.35 mm thick (see note below)
- Acrylic welding solvent

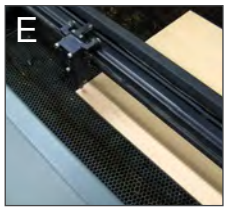
*Notes on acrylic:* Some suppliers sell "¼ inch thick extruded acrylic," while others sell "6 mm thick acrylic." In each case, the actual measured thickness of the material that is delivered can vary from 6.00 to 6.35 mm. We thus designed the components to fit together for any acrylic thickness from 6.00 to 6.3 mm. The pieces will fit a little more loosely with 6.00 mm acrylic and more snugly with 6.35 mm acrylic, but we've tested the designs successfully with both. Another consideration is that opaque / pigmented acrylic is often autofluorescent. Thus for any component that will be mounted in a microscope for fluorescent imaging (namel , the cylinder), it is important to cut the parts out of optically clear acrylic (such as McMaster-Carr #8560k355), although for illustration purposes the photos in this guide depict opaque white acrylic.

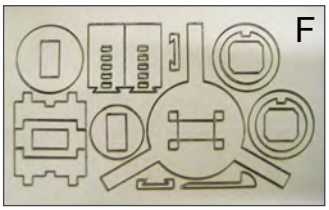
### Equipment

We used a Universal Laser Systems laser cutter, model PLS 6.75 (panel E below). Any laser cutter/engraver that can cut acrylic of this thickness will do.

### Step-by-step instructions

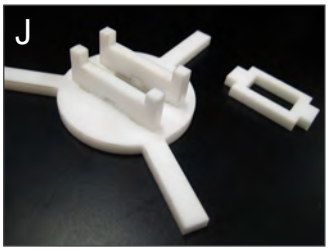
1) *Laser cut the acrylic pieces.* In Supplemental Materials S3, the file named "OMM well_all_components.dxf" has all the pieces except for the insert. The reader could use one of the user-tested inserts described in Supplemental Materials S2, or design a custom insert. Most acrylic comes with a protective backing. Leave it on during cutting (E, F, G).

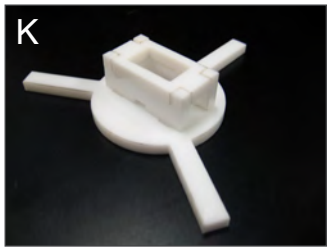





2) *Peel the backing from all pieces* (H). The remaining steps walk through how to fuse the acrylic parts together for each component.

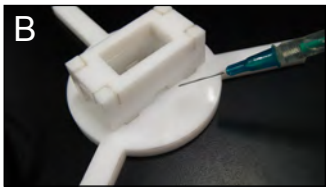

3) *Assemble the upright platform.* Gather the 4 pieces shown (I), then slot the side walls into the tripod and fuse them in place (J). Then add the top slot and fuse in place (K). Set aside.

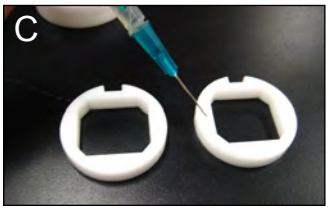




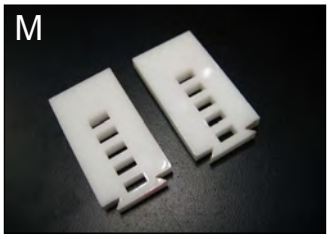

**How to fuse acrylic components together**

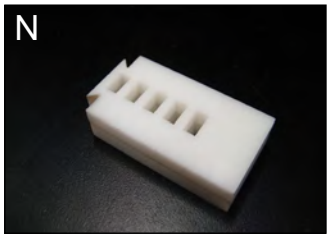
Fuse pieces together by first arranging them in the desired configuration (you can use masking tape to hold them together while fusing, although in our hands this was not necessary). Then drip acrylic welding solvent (e.g. IPS Weld-On 3 Acrylic Plastic Cement) into the joint with the supplied applicator or a polypropylene syringe with a blunted steel tip (A, B). The solvent has a very low viscosity and will easily wick into tight joints. It is not necessay to apply any pressure. When connecting two pieces that share a flat surface, another option is to apply a few drops to the flat surface of one piece, and then place the second piece on top (C, D). Assemble pieces on a disposable surface or on a piece of glass. Extra solvent will evaporate away in seconds. Pieces will be fixed in place within a minute, and fully set within a few hours.



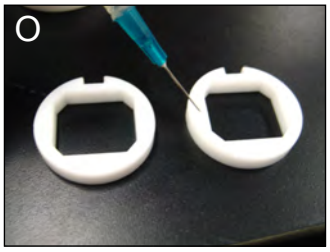


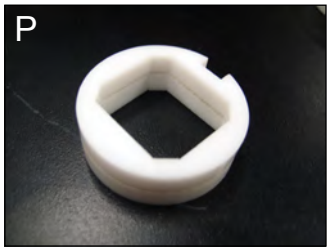
4) *Assemble the slide.* Take the two identical pieces, align them on top of one another, and then fuse them together (M, N).



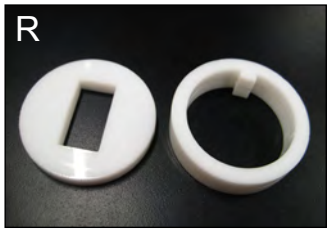



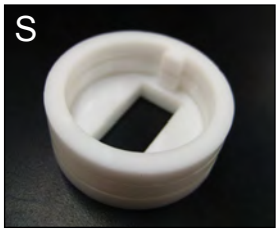

5) *Assemble the cylinder.* Take the two identical pieces, align them on top of one another, and then fuse them together (O, P).



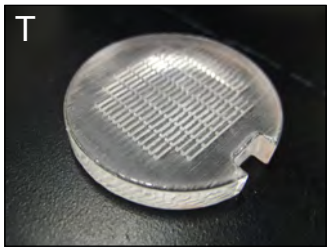



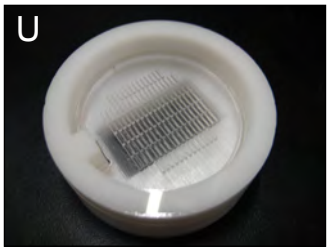

6) *Assemble the sheath.* Take the two identical ring pieces (Q), align them on top of one another, and then fuse them together. Then fuse these to the base as shown at right, with the protruding rectangular tab centered on the short end of the rectangular hole in the base (R, S).

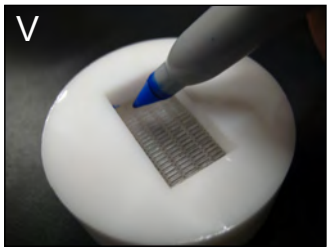



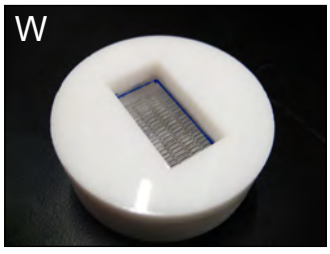



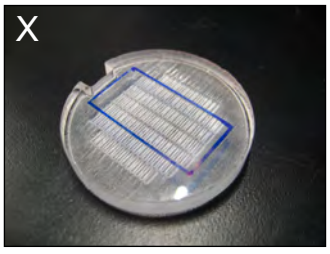

7) *Attach the slots to the back of an insert.* Once you have designed and fabricated an insert (e.g. T), you will need to attach slot pieces to the back of it. First place the insert into the now-completed sheath (U), and then trace the rectangular opening on its flat side ( , W) and set aside the sheath (X). Position and fuse two slot pieces side by side in the traced rectangle (Y).

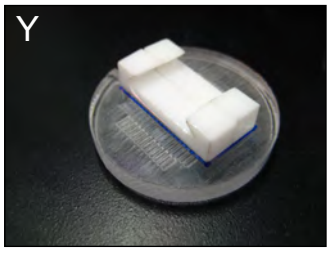


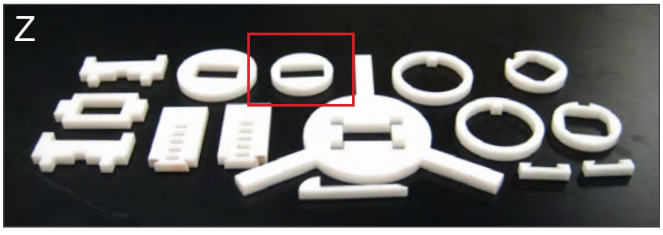




*NOTE:* The part highlighted in the red box (Z) is an optional piece called the slide lock. When using Configuration 1 (Figure 2, top), this piece can be put on the slide before connecting the insert to the slide. It then locks the insert in place.



# Supplemental materials S3: Comparison of fabrication techniques

In testing OMMAwell, we fabricated all the components using laser cut acrylic, however 3D printing is also an option. The two modes of fabrication differ in many respects, including 1) Equipment cost, 2) Choice of materials, 3) Cost of materials, 4) Speed of fabrication, 5) Ease of file design. Depending on how a user weighs these, and depending on what they have access to, each user will come to their own conclusion about which is best.

| | Equipment cost | Choice of materials | Cost of materials | Speed of fabrication | Ease of file design |
|---|---|---|---|---|---|
| Laser cut plastic | Laser cutters and 3D printers vary enormously in sophistication, resolution, and price. A new laser cutter or 3D printer that is capable of fabricating the pieces described in this article could be purchased for a minimum price of 2000-4000 United States Dollars (USD). For our tests we used a PLS6.75 laser cutter. New, this machine would cost about 20,000 USD, but it was much larger and more powerful than was needed to fabricate the components discussed in this article. | In our hands, the easiest and cheapest plastic to use for laser cutting and engraving is acrylic. Acrylic comes in clear or opaque varieties. One drawback of acrylic is that ethanol will cause cracks to form in it, eventually causing components to break down. Thus, for cleaning, it is better to use soap and water. We have not tested other plastics for engraving. | All the OMMAwell components described in Supplemental Materials S3 can be cut from a sheet of 6mm-thick acrylic that is 12 x 20 cm. We purchase acrylic from McMaster-Carr (e.g. Cat# 8560K356). The amount of material to produce all OMMAwell components costs approximately 5 USD. | Laser cutting all the OMMAwell components described in Supplemental Materials S3 takes approximately 30 minutes, plus an additional ~30 minutes to weld together the pieces.<br><br>Engraving a single mold insert takes 10 to 30 minutes, depending on size. | Laser cutting and engraving is generally done as a two-dimensional process. This means that design files can be quickly produced by anyone who can make 2D vector drawings (such as in Adobe Illustrator, Corel Draw, or Inkscape). The downside of this approach is that the complexity of the eventual 3D form is limited to shapes constructed of vertical and horizontal planes. |
| 3D printed plastic | | We have not tested 3D printed materials for OMMAwell components, but there is a wide range of options. Wittbrodt et al (2014) printed a mold for zebrafish larvae using polylactic acid (PLA). Gregory and Veeman (2013) ordered a mold for ascidians made from MicroFine Green resin, a material produced by Fineline Prototyping. Alessandri et al (2017) printed components of an imaging chamber using HTM140 resin. | This varies widely, depending on the material and provider. | This will vary depending on the 3D printer that is used, but for components of the scale of OMMAwell, printing duration will likely range from a few hours to overnight. | 3D printers will require a fully three-dimensional design file. There are several options for inexpensive or free software tools for this, as well as professional options. Each of these will have a steeper learning curve than those needed for making a 2D design, but the range of possible 3D forms is much larger. |

# Supplemental Data

Click here to download Supplementary Data S1