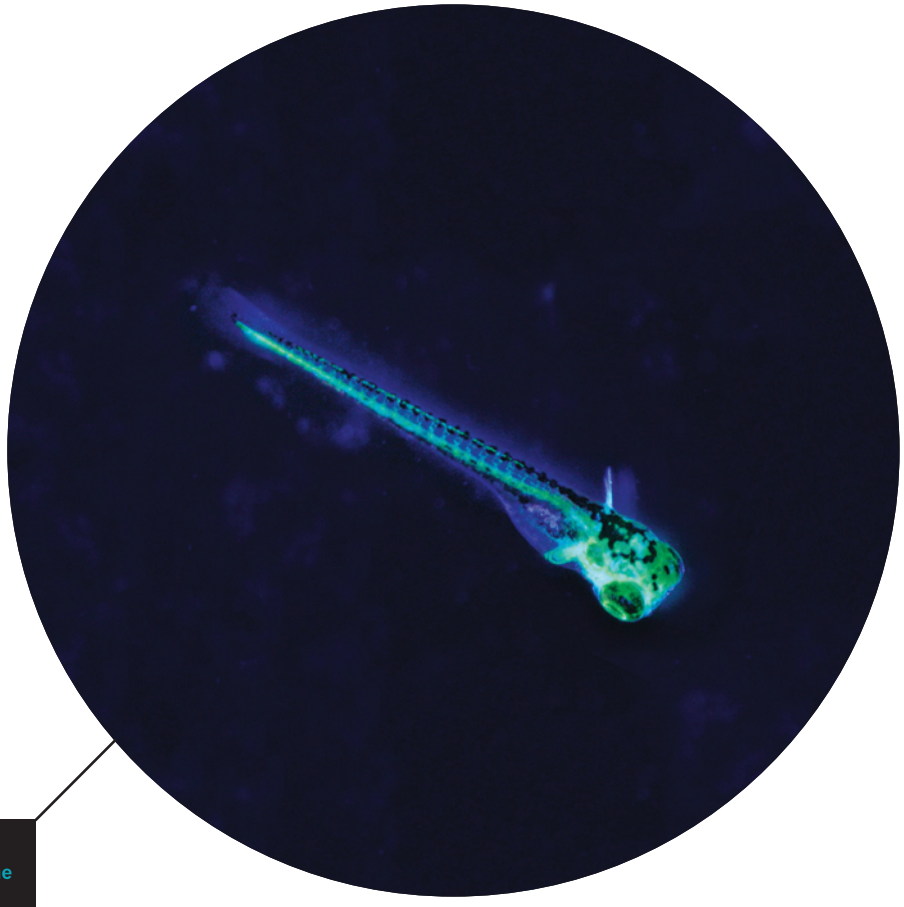

The transparency of zebrafish larvae makes the animals ideal models for seeing deep into tissues.

FISH-BOWL NEUROSCIENCE

Tiny fish trapped in a virtual world provide a window into complex brain connections.

BY VIRGINIA HUGHES

A recently hatched zebrafish is swimming upriver for the first time. Its big round eyes, bulging on the front of its eyelash-sized body, scan the surroundings. Suddenly, it sees the scenery flying forwards as a gentle current pushes it backwards. The fish flicks its tail to try to stay in place. Or so it thinks.

In reality, the baby fish is paralysed and suspended in a water-filled Petri dish by glass pipettes. The dish sits on the stage of a US$100,000 microscope in the corner of a darkened, cluttered laboratory. A film, projected from below, has transported the fish to a virtual world in which moving bands of light and dark simulate passing underwater scenery.

Although the fish doesn't move, the motor neurons that control its tail are firing away, just as if it were swimming. And when fed into a computer, those signals can control the video display, giving the fish nearly every sign that it is swimming normally. All the while, Florian Engert's microscope peers deep into the fish's tiny, translucent brain to watch

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Watch a video of a zebrafish in 'the matrix' at.
go.nature.com/lghpmp

CHARLES MAZEL/VISUALS UNLIMITED/CORBIS

neurons glow green as they fire.

Engert, the neuroscientist who developed the set-up, often jokes that the fish is just like Neo, a leading character from the 1999 sci-fi thriller *The Matrix*, in which humans have been enslaved by machines but are fed a virtual reality that leads them to believe that they are free. Engert's team at Harvard University in Cambridge, Massachusetts, hopes that the fish in this aquatic matrix will help to answer the biggest question in neuroscience: how a doughy mass of neurons in the brain gives rise to an exquisite suite of behaviours, absorbing information from the outside world and generating responses.

Since the late nineteenth century, when Spanish anatomist Santiago Ramón y Cajal pinpointed the neuron as the fundamental unit of the brain, most neuroscientists have focused on recording the electrical buzzing of individual cells. That has tended to mean sticking electrodes into the brains of cats, rabbits, rats, mice, sea slugs, squid, monkeys and even people. The approach reveals a lot about how neurons respond to inputs — such as a chemical messenger, a sound or a colour — and produce individual firing patterns, which the brain decodes to drive behaviour. But how these cells work together to translate and integrate complex, real-world sensory inputs — such as moving scenery, smells, sounds or an approaching predator — "is something that's still a big mystery", Engert says. "That's probably the main challenge for the next decade."

Larval zebrafish (*Danio rerio*) have been a workhorse model organism in developmental biology labs for about 30 years because they are cheap and relatively amenable to genetic manipulation, and have transparent tissues, allowing researchers to see inside them. Engert and a small group of neuroscientists have been looking to capitalize on these qualities to study how the brain encodes vision, hearing, movement and even fear, things that are impossible to do in the brains of more complex model organisms. And techniques such as Engert's matrix are allowing them to monitor the zebrafish's 300,000 or so neurons and to track activity in vast swathes of them simultaneously in living brains. Such innovations mean that grant reviewers and top-tier journals — which overwhelmingly favour neuroscience research in mammals — are now giving fish a chance.

"There is a sort of perfect convergence of model and methodology with zebrafish that is peaking right about now," says Joseph Fetcho, a neurobiologist at Cornell University in Ithaca, New York, who pioneered brain-circuit research in the fish. This impressive array of tools, he adds, makes him wonder "why one would use any other model for basic questions about circuits and behaviour".

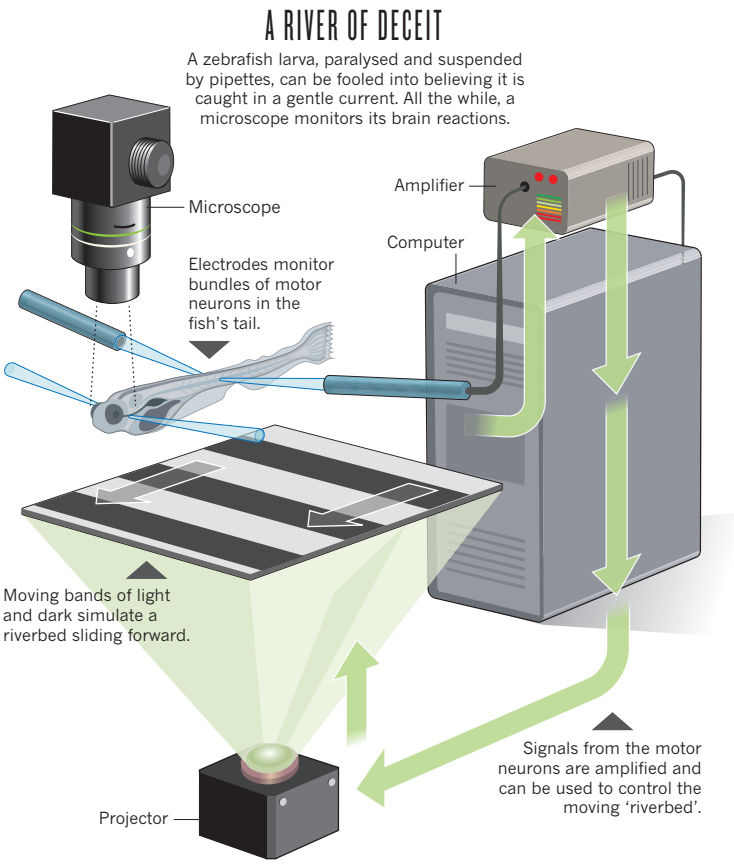
SPOTLIGHT AQUARIUM

During his early career at Stony Brook University in New York, Fetcho worked on goldfish brains until he got frustrated recording from only a couple of cells at a time. He switched to zebrafish in the mid-1990s, after two strikes of serendipity.

First, at a zoology conference, Fetcho stumbled into a workshop on using zebrafish in high-school biology classes. He realized how easy it was to watch their translucent embryonic cells divide and, over the course of just a few days, develop into organs and limbs.

Second, he came across a paper describing how to fill neurons with a green dye that is sensitive to calcium[1]. Because neuronal firing requires an influx of calcium ions, this method provided a way to view cells in action. The paper used cells isolated from chick spinal cords, but Fetcho thought the same approach could light up the neurons of live zebrafish. He went to a local pet shop and bought a mating pair. The next day, he had fertilized eggs to experiment with.

In Fetcho's first zebrafish paper[2], published in 1995, he and his colleague Donald O'Malley used the calcium-sensitive green dye to track the activity of motor neurons during a predator-escape reflex, which is triggered by poking the fish in the head. His team went on to show that neurons in different segments of the hindbrain encode how the fish turns its body to escape from a predator[3]. The researchers then created the first transgenic line of fish to express a calcium indicator, similar to the green dye, in all neurons, so that the dye no longer had to be injected[4].



NIK SPENCER/*NATURE*

Neuroscientists also started to use calcium indicators to label neural circuits in other animals. In a landmark study in 2001, for example, researchers mounted miniature two-photon microscopes — which can probe more than a millimetre deep into tissues — on the heads of scampering rats to reveal the firing patterns of many individual neurons at once[5]. Another group observed the brain of a fruitfly that was secured under a microscope while its legs walked freely on top of a polystyrene ball[6].

But when using rodents or flies, researchers must first cut windows in the animals' heads to expose the part of the brain they want to image. And even then, the microscopes can probe only superficial layers of these opaque brain tissues.

Only two popular model organisms have small, transparent brains and can be easily genetically engineered: the larval zebrafish and the soil nematode *Caenorhabditis elegans*, which has just 302 neurons. But to zebrafish researchers, *C. elegans* is too small and its circuits are too simple. Its brain is also covered in a tight cuticle and its neurons are tiny, making it difficult to make recordings with conventional electrodes. Besides, the worms don't show the same variety of behaviours — notably, those that require sophisticated vision — that a fish does. "We could probably do similar things in *C. elegans*," says Engert, "but I'd be worried that the answers aren't as interesting."

When Engert launched his Harvard lab in January 2002, he was determined to focus on the circuitry of larval zebrafish. His former adviser's father had been close friends with Nobel-prizewinning developmental biologist Christiane Nüsslein-Volhard, who helped to pioneer the zebrafish as a model for embryonic development. Still, Engert had never seen one in the flesh. "I was quite shocked when I saw one for the first time; they're so small," he says.

It was an audacious choice, but it fit Engert's personality and penchant for risk. He's known around the Harvard biology building for never

wearing shirtsleeves, rollerblading into lectures and riding his motorbike without a helmet. A native German, he did his Harvard tenure talk in lederhosen (he earned tenure in 2009). Last year, he was nearly trapped under an avalanche while skiing off piste in Austria — shirtless.

At Harvard, it took Engert a couple of years to set up his lab experiments for zebrafish. At first, he tried imaging neurons while the fish were swimming freely. But "their whole brain wiggles", he says, making imaging impossible. That's when he started to build the virtual environment.

For a study published last May[7], Engert's postdocs Ruben Portugues and Misha Ahrens built a simple virtual world consisting of red and black stripes that moved under the fish. This visual stimulus, crude as it is, was enough to make the animals feel as though they were being swept backwards by a rushing river, and send out muscle commands to push forwards.

With the stroke of a few computer keys, the researchers can manipulate the scene, making the stripes go slower or faster. The tweaks make it seem, to the fish, that its movements have been either too weak or too strong, so it makes adjustments to stay in place. This behaviour is called motor adaptation, and it is akin to what people do when they're walking and suddenly slide on a patch of ice, for instance. The brain takes in the new environmental information and adjusts movements to prevent a fall.

Studies in monkeys had revealed that specific neuronal populations were involved in motor adaptation. "If something happens that's unexpected, it needs to get processed completely differently from if it's expected," Engert says. The processing "either tells me that something is happening in the world outside, independent of my motion, or it tells me there's something wrong with my body".

His fish study implicated the same populations of cells, but also showed something new. Certain neurons within these populations encode high-feedback gains — that is, visual feedback telling the fish that its muscles are stronger than expected — whereas others respond to feedback that its muscles are weaker than expected.

This is the sort of nitty-gritty detail that neuroscientists, hoping to look at the individual neurons within circuits, relish. In the past few years, other labs have revealed similar details. In 2010, for example, researchers in Japan pinpointed specific neurons in the habenula — a deep brain region that is difficult to study in mammals — as crucial players in the zebrafish's response to fear[8]. In 2011, Fetcho showed that neurons in the fish's hindbrain are neatly stacked during development in a way that allows the oldest cells to drive the fastest movements and younger cells to control more refined motions[9].

But researchers are less excited by Engert's results than by his technology, which allows them to view every neuron in an entire living, working brain. "You can't do it in any other animal," says Martin Meyer, a neuroscientist at King's College London who used calcium imaging to show how different layers of cells in the zebrafish brain respond to objects moving in specific directions[10]. "There's more or less endless scope, once you have that set up."

Neuroscientists who work on other animals also applaud Engert's technique, although they have some reservations. "It doesn't give you everything that there is to know," says Rex Kerr, who studies nematode brains at the Howard Hughes Medical Institute's Janelia Farm Research Campus in Ashburn, Virginia.

Kerr notes that the two-photon microscope can't actually image all 300,000 neurons at once. Instead, Engert's group systematically monitors 1,000 or so neurons in 300 subregions, from a total of 32 fish, and then uses a computer model to merge the activity onto a reference brain. For some behaviours, this averaged neural activity can mask interesting activity patterns in individual neurons, Kerr says. Still, a lot of interesting activity is determined by populations of many cells. Zebrafish are "extremely valuable" for looking at those ensembles, he says.

FISH FUTURES

There are practical reasons why more neuroscientists haven't worked on zebrafish. Many sophisticated behaviours — such as communication, social interaction and complex emotions — aren't displayed by the animal at all. And scientists have yet to develop ways to study even some basic reflexes in the fish.

That means it is not yet clear what other behaviours Engert's technique will be able to test. Jason Rihel, who is setting up a lab at University College London, would like to use a similar approach to study neurons that produce hypocretin, which are involved in sleep and wakefulness. "If we could watch the whole brain while we're tickling the hypocretin neurons, or inhibiting them, then we might be able to map out every neuron in the brain that has altered activity," Rihel says. He is a bit worried, however, that the immobilization will affect the fishes' slumber.

JUSTIN IDE/HARVARD UNIV.

"WE'LL HAVE A WIRING DIAGRAM OF THE WHOLE BRAIN THAT CAN RELATE STRUCTURE TO FUNCTION."

FLORIAN ENGERT

Engert, in typical fashion, has ambitious plans. He's got each of his five postdocs and about eight graduate students working on a different zebrafish experiment, from uncomfortably warm baths that test fear-learning to alcohol-rich waters to look at the effects of positive rewards.

He is also working on a "side project" that is likely to get a lot of attention later this year: the zebrafish connectome. Engert's team is doing whole-brain functional imaging of live, baby fish while they look at moving bars, and then passing those brains on to Harvard colleague Jeff Lichtman, who will use an electron microscope to trace the anatomical connections. "We'll have a wiring diagram of the whole brain that can relate structure to function," Engert says.

With these types of resource, Engert and his zebrafish might even find what neuroscientists have been searching for since Cajal: a fundamental principle that describes how circuits interact with one another. "My life will not be a failure if it doesn't happen, but I'd love to find it," Engert says. "And it's ten times more likely to happen in fish than mice." ■

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