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The first chick brain with non-invasively embedded beads: a foundation for the automation of brain research

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Abstract

Background The automation of biotechnology, such as next-generation DNA sequencing, revolutionarily provides massive amounts of data and integrates various research fields. By contrast, many non-automated brain research fields are not interconnected with one other. In this study, we developed a basis for the automation of brain research. Two major technical barriers for the automation of brain research in vertebrates are the necessity for skull incision and a precise inoculation system for probes, devices, and electrodes in defined brain locations.

Results The former barrier in the background was overcome by inoculating probes into the future brain area of chick embryos before skull formation. Fluorescent micro-beads that mimic probes were inoculated into the future brain area of chick embryos, and 20% of the manipulated embryos hatched, with 71% of the hatched chicks containing multiple beads in their brains.

Conclusions With this technique, beads are embedded inside the brain without skull incision, promising a novel non-invasive method that overcomes the drawbacks associated with traditional invasive brain manipulation.

Keywords Near-infrared, Non-invasive, Brain, Optogenetics, Automation, Behavior, Development

Background

In order to facilitate studying vertebrate behavior, we will propose here the concept on automation of brain research.

Sanger and Mullis manually operated DNA sequencing and PCR, respectively (Additional file 1: Fig. S1a). Automatic DNA sequencing technology combining their principals has been developed (Additional file 1: Fig. S1b), leading to the completion of various genome projects on model organisms. Furthermore, next-generation sequencing based on non-Sanger methods has generated massive DNA sequences of many organisms and individuals (Additional file 1: Fig. S1c), revolutionarily expanding and integrating numerous biological research fields.

By contrast, an integrated approach to brain research is lacking because a standardized automated system has not been established yet (Fig. 1). With the aim to automate brain research, a robot was developed that could implant 3072 electrodes, mimicking a MEA (multielectrode array), into the rat brain (Fig. 1(a)). In total, 96 threads (each containing 32 electrodes) are implanted, with a rate of six threads per minute (Musk 2019). As such, it takes 16 min to complete this robotic surgery.

Instead of this invasive robotic approach, we here propose a non-invasive automated system using vertebrate embryos. This approach is based on the inoculation of probes into the future brain area of developing chick embryos before skull formation. After the manipulated chicks hatch, the probes in their brains can communicate with helmet transmitters sending and

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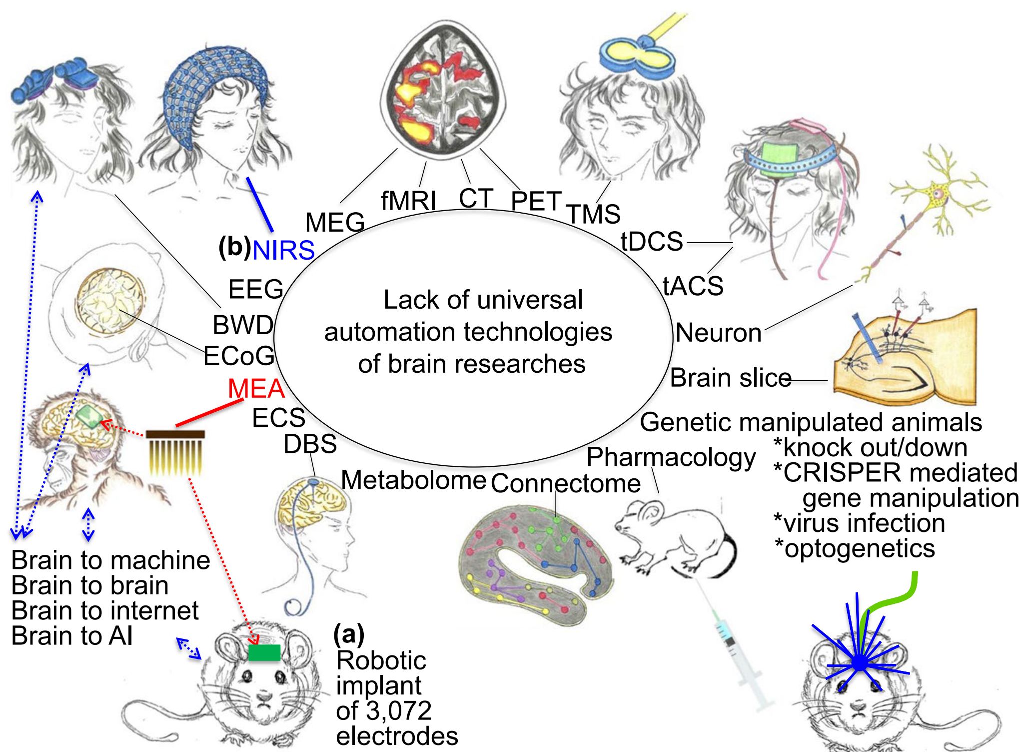


Fig. 1 Lack of automation in brain research. The various methods in brain research described below are not automatized or technically interconnected with each other. Two approaches exist in vivo: non-invasive and invasive approaches. Non-invasive technologies, such as positron emission tomography (PET), computed tomography (CT), functional magnetic resonance imaging (fMRI), magnetoencephalography (MEG), near-infrared spectroscopy (NIRS), and electroencephalography (EEG), measure brain activities via different principals (Rothschild 2010), whereas transcranial magnetic stimulation (TMS), transcranial direct current stimulation (tDCS), and transcranial alternating current stimulation (tACS), broadly stimulate the brain (Miranda 2013). These non-invasive technologies are not interconnected and cannot evaluate or stimulate individual neuronal activity. Genetically manipulated animals, including knockout, knockdown, or overexpressed mice, and pharmacological approaches are also categorized as in vivo non-invasive brain research. Conversely, electrocorticography (ECoG), an in vivo invasive technology, simultaneously stimulates and records broad areas of the brain cortex (Rothschild 2010). Other in vivo invasive approaches, such as microelectrode arrays (MEA), electrical cortical stimulation (ECS), and deep brain stimulation (DBS), enable the direct stimulation and recording of neuronal activities (Rothschild 2010). Optogenetic manipulation is categorized as in vivo invasive technology that directly stimulates or suppresses neuronal activities, because blue light, which is necessary for optogenetics, must be supplied under the skull incision (Chen et al. 2018). Of note, digital data obtained by ECoG or MEA can be interconnected with data from other animals and robots via the Internet (Lebedev et al. 2005). Furthermore, the brown wireless device (BWD), a wireless broadband intracortical brain-computer interface (iBCI), non-invasively records and decodes broadband field potentials and spiking activity from individuals with tetraplegia (Simeral et al. 2021). Various in vitro brain research methods exist, including constructing the connectome (Winnubst et al 2019), stimulating, suppressing, and detecting the activities of neurons and brain sections via electrodes or pharmacological methods, dissecting signal transduction inside neurons and brain sections, and various biochemical analyses (of DNA, RNA, proteins, and metabolites, including neurotransmitters). (**a**) 3072 electrodes mimicking MEA are robotically implanted into the rat brain (Musk 2019). (**b**) NIRS is utilized for functional brain imaging. Near-infrared (NIR) penetrates into human brain tissues through the skull (Rothschild 2010)

receiving near-infrared (NIR) light from outside the skull (Fig. 2(a)), similar to the existing near-infrared spectroscopy employed in humans (Fig. 1(b)).

Numerous NIR-driven probes for brain research have already been developed, including probes that emit blue light to stimulate optogenetically manipulated neurons (Chen et al. 2018), sense catecholamine (Beyene et al. 2019), record neuronal activity (Liu et al. 2020), stimulate neurons (Ma et al. 2019), suppress neurons (Lee et al. 2018), and deliver drugs as well as release a variety of macromolecules (Li et al. 2015) (Fig. 2). However, currently, all these probes must be invasively and manually inoculated into the brain. The development of NIR-driven brain micro-probes could be accelerated if hundreds of probes could be automatically, accurately, reproducibly, and non-invasively embedded into the brain.

We theoretically present a possible automatic inoculation method of hundreds of probes into the brain of chick embryo, as follows (Fig. 3). Four existing industrial technologies (Fig. 3(a)–(d)) can be combined to create a robot that automatically inoculates individually

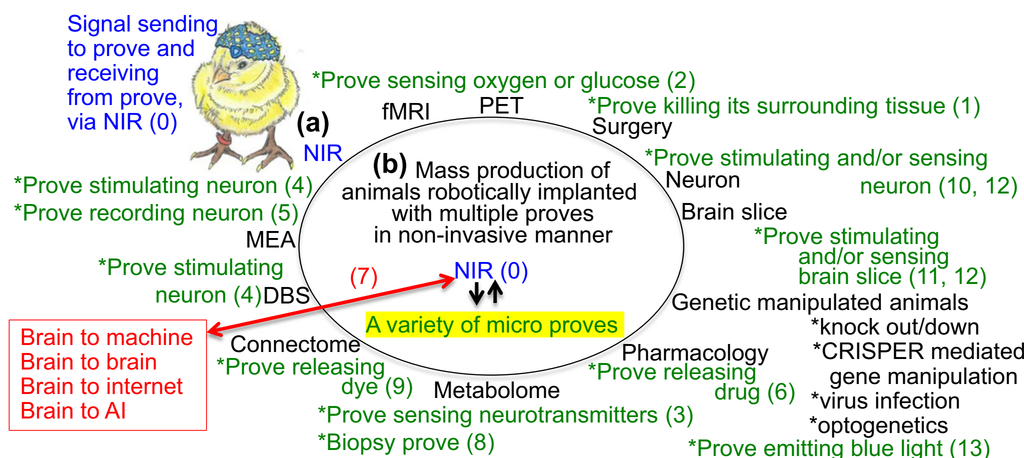


Fig. 2 The key concepts of our research -Our vision for the automation of brain research. An illustration of a chick, whose brain contains probes, wearing a blue helmet representing a near-infrared (NIR) transmitter (0), as in Fig. 1b. (**a**) NIR light should reach the deep brain of chicks (Chen et al 2018). (**b**) If hundreds of probes, devices, and electrodes are embedded in a defined area of vertebrate brain, the probes could individually communicate with the outside of the skull via NIR (0). Some existing brain research in Fig. 1 can be replaced with the NIR-driven probes (1)–(13), as follows. We propose candidate probes and integrating whole brain research. Some existing methods in brain research (Fig. 1) can be replaced with the technology of non-invasively embedding brain probes that are individually driven by NIR (0) (**a**). Such technologies (1)–(13) include probes that (1) destroy the surrounding neurons in a temporally and spatially specific manner, like surgical resection; (2) sense oxygen or glucose, like fMRI or PET; (3) sense a variety of molecules including neurotransmitters, (4) simultaneously or individually stimulate or suppress neuronal activities, like MEA; (5) simultaneously or individually record neuronal activities, like MEA; and (6) deliver drugs in a temporally and spatially specific manner, instead of drug injection. Furthermore, (7) massive digital signals of a particular animal that are interconnected with machines, robots, and other animals via the internet according to the brain-net concept could be employed. Upon such detailed in vivo top-down probe analyses (1–7), (8) biopsies of the corresponding probes can enable a variety of biochemical analyses, and (9) the neuronal network as a connectome (Winnubst et al. 2019) can be visualized by neurons that take up retrograde-type dyes released from the probes. Furthermore, after euthanizing animals, (10–11) neurons and brain sections can be analyzed in vitro by stimulating, suppressing, and recording their activity via the probe; (12) and the signal transduction of neurons and brain sections can be analyzed in vitro around the probe. These bottom-up analyses (8–12) could be functionally and mutually linked to top-down analyses (1–7), integrating all brain research. Finally, when using transgenic animals or recombinant virus-infected animals for optogenetics, (13) blue light can be supplied from the NIR-driven probe (Chen et al. 2018)

controllable probes into the head region of developing chick embryos, resulting in a mass production of chicks with non-invasively embedded probes. The position of inoculated probes in an embryo (Fig. 3(c), (g)) and in the brain of a hatched chick (Fig. 3(e)) can be precisely recorded. Both positions can be determined by deep learning artificial intelligence (AI) (Fig. 3(f)), enabling the mass production and reproducibility of implanted probes in defined regions of chick brains. The system described above will be called "Spemann" (Fig. 3(h)). The development of "Spemann" could trigger the explosive development of various near-infrared (NIR)-driven probes.

Question is whether chicks hatch upon inoculation of proves during embryonic development (Fig. 4(a), (b)). If such chicks hatch, do they carry proves in their brain? (Fig. 4(c)). Here, we inoculated fluorescent micro-beads that mimic brain micro-probes into the head regions of chick embryos (Fig. 4), thus obtaining the first animals with non-invasively inoculated multiple beads in their brains at birth (Figs. 5, 6). The application of our successful technique in integrating brain research and applying to study a variety of animal behaviors, is presented (Fig. 7).

Methods

Preparation of fluorescent beads

Fluorescent Nile Red Particles (size: 5.0–7.9 μm; FP-6056-2) were purchased from Spherotech. Bead suspensions (100 μL) were mixed with 1 mL of sterilized phosphate-buffered saline (PBS). The mixture was centrifuged, and the supernatant was discarded. Then a needle was soaked in the remaining sterilized dense bead suspension for bead attachment (Fig. 4(a)).

Inoculation of fluorescent beads into chick embryos

Fertilized chicken (*Gallus gallus*) eggs were purchased from the Yamagishi poultry farm (Gunma, Japan). The fertilized eggs were wiped once with 70% ethanol and incubated at 37.6 °C (humidity 65 ± 5%) in an incubator (PH-3; Showa Furanki) until reaching the developmental stages 11–18 (Bellairs and Osmond 2005). During incubation, fertilized eggs were manually rotated twice per day. After reaching the appropriate developmental stages,

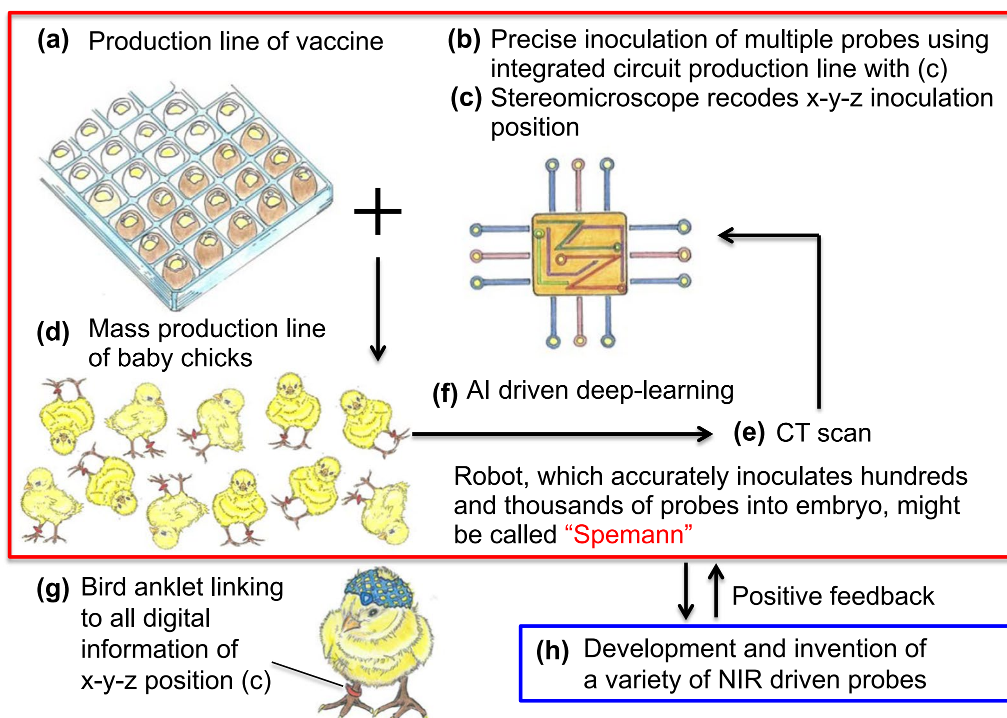


Fig. 3 The necessity to develop the robot "Spemann". We propose how to automatically inoculate probes into chick brains. To achieve accurately embedding the beads in specific regions of the brain, it is essential to develop automatic robotic inoculation systems (Fig. 2b). The following four different industrial systems (**a**–**d**) exist: (**a**) the traditional pharmaceutical vaccine production line (which automatically sterilizes fertilized chick eggs and bores a hole in the eggshell), an automatic precise probe inoculation system combining (**b**) an integrated-circuit electronic production line and (**c**) an automatic staging and recording stereomicroscope system (utilized in various biological and medical sciences to record digital information of the x_y_z position of the object), and (**d**) poultry farming for the massive production of chicks. Combining these industries will enable a mass production line of chicks whose brains contain hundreds of beads (probes) (Fig. 2b). Moreover, (**e**) computed tomography scanning of chick brains could individually, precisely, and digitally record the x_y_z position of each embedded bead in the brain. Finally, (**f**) artificial intelligence-mediated deep learning of the inoculation position in the embryo (**c**, **g**) and the real position of the beads embedded in the brain (**e**) guarantees an optimized program that inoculates probes into pre-defined brain regions. Since existing surgical robots have names such as "da Vinci", the entire system of this putative automatic robot could be called "Spemann", referring to the developmental biologist who manipulated vertebrate embryos and found "the organizer" (Spemann and Mangold 2001). "Spemann" could meet the academic and industrial demand for brain probes, devices, and electrodes that are controlled via NIR from outside the skull ((**h**) and Fig. 2A(a))

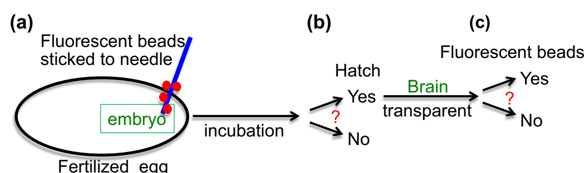
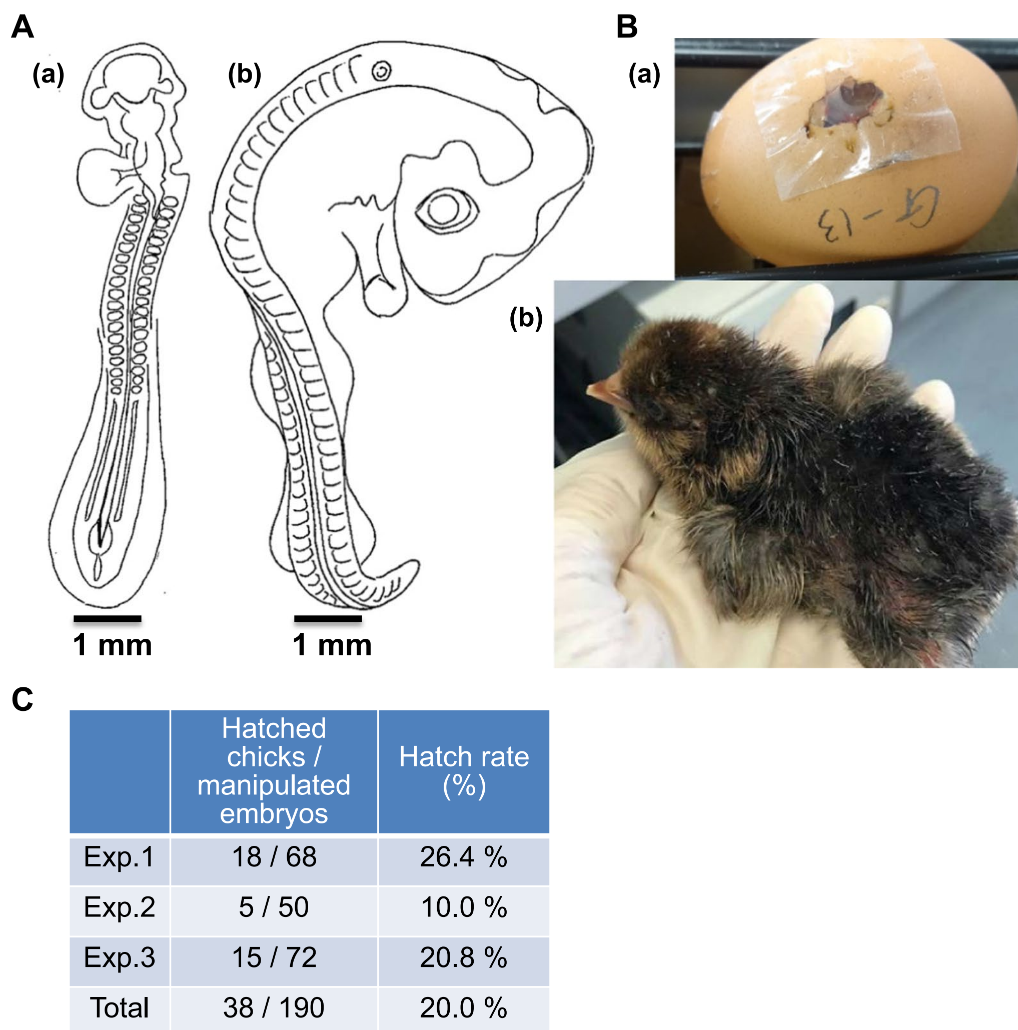


Fig. 4 The aim of this study. (**a**) Fluorescent beads mimicking micro-probes were inoculated into the future brain region of chick embryos. (**b**) After incubating the manipulated eggs, we examined whether they would hatch. (**c**) Upon hatching, the whole brain was removed from the skull. The brains were treated with transparent reagent and examined for fluorescent beads

a window (diameter, 5 mm) of eggshell was cracked open. After removing 0.7 mL of egg white, the head area of the chick embryo was stabbed with a needle (React-system, 35G) coated with fluorescent beads. After bead implantation, the window of eggshell was sealed with adhesive tape (Nichiban No. 405-1P). The manipulated embryos were incubated (at 37.6 °C and 65 ± 5% humidity) in a fully automatic egg incubator (Maxi II EX; Brinsea Products Inc), which automatically and intermittently rotates manipulated eggs, until the 16th day. Finally, manipulated eggs were transferred to the PH-03 incubator (at 37.6 °C and 65 ± 5% humidity) and incubated without egg rotation, from the 17th day to their hatching.

Transparent treatment of the whole brain

The hatched chicks were euthanatized by exposure to isoflurane, the occipital region of the skull was incised, and the whole brain was removed and placed into a 12-well plate (Greiner Bio-One). The brain was soaked in 4% paraformaldehyde in PBS (Wako) at 4 °C overnight. The fixed brain was washed three times with PBS and soaked in



	Hatched chicks / manipulated embryos	Hatch rate (%)
Exp.1	18 / 68	26.4 %
Exp.2	5 / 50	10.0 %
Exp.3	15 / 72	20.8 %
Total	38 / 190	20.0 %

Fig. 5 Manual implantation of fluorescent beads into the head area of chick embryos. **A** Stage-13 and -18 embryos. Chick embryos at stages 13 (a) and 18 (b), taken from the textbook (Bellairs and Osmond 2005). **B** A photograph of one of the first hatched chicks after inoculation. (a) After implanting the micro-beads into the embryos, the eggshells were sealed with tape. Then the manipulated embryos were incubated until hatching. (b) The first animal to have micro-beads in its brain at birth. **C** The hatch rate of the inoculation experiments. Results of three independent experiments using stage-18 embryos

transparent reagent (CUBIC-L; Tokyo Kasei), according to the manufacturer's protocol. CUBIC-L was changed twice within 48 h and then changed every 48 h for 6 days. Afterwards, the brain was washed three times with PBS and then soaked in 50% CUBIC-R (Tokyo Kasei; the original solution was diluted with distilled water) at room temperature overnight. Finally, the brain was soaked in 100% CUBIC-R for 24 h. The transparent whole brain was examined under a fluorescent stereomicroscope (Leica MZ10 F).

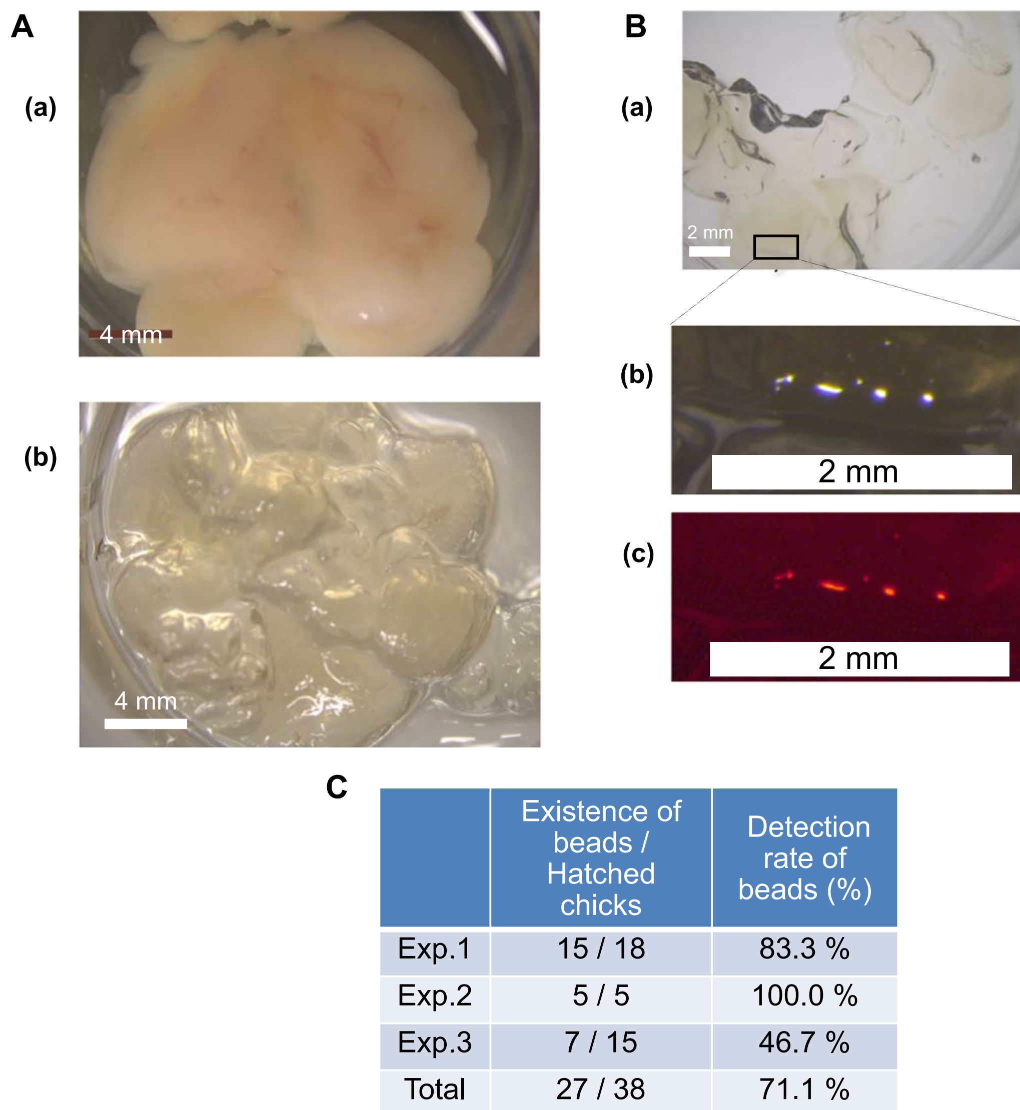
Results

Developmental stages of chick embryos

The future brain area of chick embryos is visible at developmental stage 10, and the hard skull forms by day 13 (Bellairs and Osmond 2005). Since the formation of the vascular network between the embryo and yolk after stage 18 hinders inoculation experiments, beads must be inoculated before stage 18 (see stages 13 and 18 in Fig. 5A(a) and (b), respectively). Since no commercially available brain probes, devices, or electrodes that are suitable for inoculating chick embryos exist, we inoculated 0.4–50 μm sized red or green fluorescent beads, instead of probes.

Inoculation of chick embryos at stages 11 and 13 is fatal

Chick embryos at stages 10–11 might be appropriate for the inoculation of beads because the brain primordium was shown to be swapped between males and



	Existence of beads / Hatched chicks	Detection rate of beads (%)
Exp.1	15 / 18	83.3 %
Exp.2	5 / 5	100.0 %
Exp.3	7 / 15	46.7 %
Total	27 / 38	71.1 %

Fig. 6 Fluorescent beads in the head area of hatched chick. **A** Transparent treatment of the whole brain. Photographs of (a) the whole brain fixed with 4% paraformaldehyde and (b) the transparent whole brain. **B** Multiple beads were detected in the brains. Fluorescent beads in the transparent brain tissue were photographed; (a) a large part of the whole brain (bright field), (b) the restricted region (open rectangle in (a)) (relief contrast), and (c) the same region as in (b) (fluorescence). **C** The number of brains with beads among the hatched chicks. In three independent experiments (Fig. 5C), the numbers of manipulated brains with fluorescent beads were counted

females at stage 10 (Maekawa et al. 2013). We initially used embryos at stages 11 and 13 for bead implantation. Since the head regions of embryos at stages 11 and 13 were still small (Fig. 5A(a)), they had to be visualized by a sub-blastodermal injection of black ink (Maekawa et al. 2013). Under a stereomicroscope, the head regions of the embryos were stabbed with 33G needles (outer diameter, 0.25 mm) coated with 50 µm fluorescent beads (Fig. 4(a)). The manipulated embryos were then incubated; however, they all died before hatching ("NO" in Fig. 4(b)).

Stabbing the needle (outer diameter, 0.25 mm) into the head region (width, 1 mm; Fig. 5A(a)) of embryos at stages 11 and 13 was speculated to lethally damage the embryos. Furthermore, the 50 µm fluorescent beads were probably too large and thus unsuitable for implantation at embryonic stages 11 and 13. Although the exact causes of death were uncertain, we simultaneously changed the following three experimental conditions: embryonic stage, needle size, and fluorescent bead size.

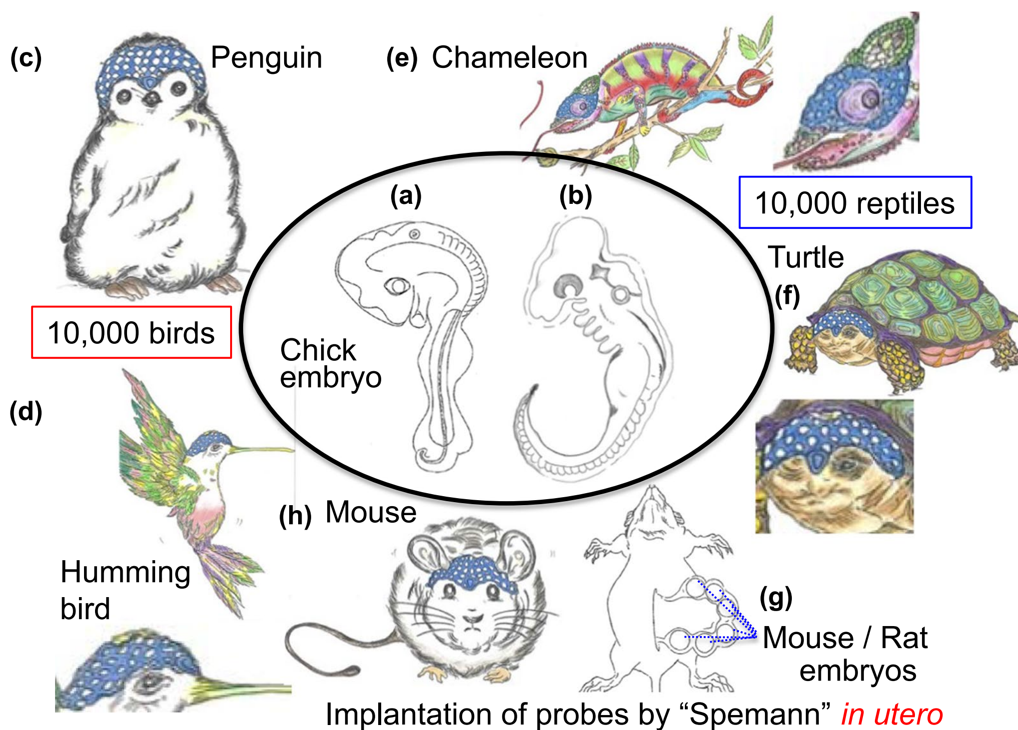


Fig. 7 Application to amniotic embryos. **a** Stage-18 chick embryos (opposite side view of Fig. 5A(b)) utilized for bead inoculation in this study. (**b**) Sketches of Haeckel's pharyngula-stage chick embryo (Richardson 1995). Illustrations of (**c**) a penguin, (**d**) hummingbird, (**e**) chameleon, and (**f**) turtle. (**g**) The abdomen of small mammals (e.g., mice and rats) can be robotically opened, and then probes can be inoculated into the head region of embryos in utero by "Spemann". (**h**) An illustration of a mouse whose brain has multiple probes. All animals (**c**–**f**, **h**) are wearing a blue helmet representing a NIR transmitter, as in Fig. 1(b) and Fig. 2A(a)

Chicks from embryos manipulated at stage 18 successfully hatched

The tip of the 35G needle (outer diameter, 0.23 mm) coated with 5.0–7.9 μm sized fluorescent beads was stabbed into the future brain area of stage-18 embryos without using a stereomicroscope. Since the head regions of stage-18 embryos were large enough to be recognized by the naked eye (Fig. 5A(b)), injecting black ink for visualization was unnecessary. After inoculation (Fig. 5B(a)), embryos were incubated until they hatched, and 26.4% of embryos hatched (Fig. 5C, Exp. 1). A photograph of one of the hatched chicks is shown (Fig. 5B(b)). Two additional independent experiments using stage-18 embryos yielded hatch rates of 10.0% and 20.8% (Fig. 5C, Exp. 2 and 3). A total of 38 hatched chicks out of 190 manipulated embryos were obtained ("YES" in Figs. 4(b) and 5C). The average hatch rate of the manipulated embryos was thus 20.0%.

The chicks had beads inside their brains at birth

To examine the presence of inoculated beads in the brains of hatched chicks (Fig. 4(c)), all 38 hatched chicks were euthanatized, and their brains were removed. Each whole brain was fixed with paraformaldehyde (Fig. 6A(a)) and then soaked in transparent reagent (Fig. 6A(b)). To detect the fluorescent beads, the transparent brains were observed under a fluorescent stereomicroscope (Fig. 6B(a)). We detected multiple beads in a small, restricted region of the brain (Fig. 6B(b), (c)).

Among the 38 hatched manipulated chicks, 27 chicks had fluorescent beads in their brains (Fig. 6B, "YES" in Fig. 4(c)). The average rate of bead detection was 71.1%. Of note, multiple beads were always detected (Fig. 6C(b), (c)), reflecting the unlikelihood of picking up only one bead with the needle. In other words, dozens of beads mimicking micro-probes can be simultaneously embedded into chick embryos. Similar results were obtained with 0.4–0.6 μm sized fluorescent beads (Additional file 1: Fig. S2).

Discussion

Since first manual experiments on DNA sequencing and PCR were reported, industries developed automatic-machines based on original principal (Additional file 1: Fig. S1).

Two major technical barriers for the automation of brain research in vertebrates exist. The first is the necessity for skull incision. This was practically solved in this

study (Figs. 4, 5, 6).The second technical barrier is the necessity for precise and reproducible inoculation of probes, devices, or electrodes in a defined brain region. This could be also theoretically solved in this study (Figs. 2, 3). The practical and theoretical solutions presented in this study could contribute to achieving automated brain research and hold promise for the critical transition from traditional invasive brain manipulation to non-invasive approaches. Such automated-brain researches will be developed by industries, just like automatic-DNA sequencing.

Importantly, a putative "Spemann" system (Fig. 3) could reproducibly and simultaneously embed multiple probes in the brain, enabling the reproduction of any experiment, anytime and anywhere. Furthermore, all digital data could be opened, shared, analyzed, and utilized by everyone, similar to the free accessibility of existing DNA sequence data banks (Additional file 1: Fig. S1). In a similar manner to the recent establishment of automatic DNA sequencing (Additional file 1: Fig. S1b, c), positive feedback between continuous technological improvements that accurately inoculate brain probes and the development of various functional probes (Fig. 3(h)) will synergistically accelerate and integrate a variety of top-down and bottom-up brain research (Fig. 2). Once such positive feedback begins (Fig. 3(h)), big data and the industrialization of neuroscience (Frégnac 2017) will be accelerated under Moore's law. Thus, the experimental results in Figs. 5, 6 will be a key foundation for the transition from the brain research in Fig. 1 to that in Fig. 2, via both the development of "Spemann" system (Fig. 3) and new functional probes (Figs. 2, 3(h)). If so, we predict that the "emperor" of neuroscience research (Yartsev 2017) will wear a "new wardrobe" (i.e., the chick as a model (Rogers 1992)) in the near future.

Finally, we propose application of the technology on chicks toward studies on a variety of vertebrate behavior. This method of producing chicks with multiple probes embedded in the brain in a non-invasive manner could be applied to any amniote. In chick embryos, stage 18 (Figs. 5A(b), 7(a)) is similar to the pharyngula stage (Fig. 7(b)), sketched by Haeckel nearly 150 years ago (Richardson 1995). The pharyngula embryonic stage of reptiles and mammals is remarkably similar to the embryo in Fig. 7(b), noted by Haeckel (Richardson 1995). Thus, the proposed automatic "Spemann" system (Fig. 3) could be applied to any chick-type eggs derived from birds and reptiles. Thus, the behaviors and brain activities of many animals, such as penguins, hummingbirds, chameleons, and turtles (Fig. 7(c)–(f)), could be analyzed (Fig. 2), tremendously facilitating comparative brain research. A robot embedding multiple electrodes in the rat brain has been developed (Fig. 1(a)) (Musk 2019). Thus, a robot that could operate small mammals (e.g., mice and rats) and embed probes into the head region of embryos in utero could be developed (Fig. 7(g)) using the "Spemann" method, yielding mice or rats with probes inside their brains at birth (Fig. 7(h)).

Conclusions

With the technique developed here (Figs. 5, 6), beads are embedded inside the brain without skull incision, promising a novel non-invasive method that overcomes the drawbacks associated with traditional invasive brain manipulation. Such novel animals with beads in their brains at birth will open completely new avenues toward the automation of brain research (Figs. 2, 3, 7), leading to enormous applications on studying a variety of animal behaviors (Fig. 7). Beside brain researches, the automation of developmental biology could also be achieved with the "Spemann" method (details in Additional file 1: Fig. S3 and its legend). Thus, our proposal in this study could accelerate all brain researches, studies of a variety of vertebrate behavior, and studies of developmental biology in many amniotes.

Supplementary Information

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Additional file 1. Fig. S1. Automation of the next-generation DNA sequencing (NGS) is essential in a variety of biological research. **Fig. S2.** Multiple 0.4-0.6 μm sized beads were detected in the brain. **Fig. S3.** Automated approaches to understand the evolution and development of animals' body plan.

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Author contributions

MS designed the study. AY conducted the experiments and the data analysis. AY and MS wrote the manuscript. All authors have read and approved the manuscript.

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Not applicable.

Availability of data and materials

Since all data we have are shown in main and Additional file 1, there is no data deposition along with the manuscript. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All experiments were carried out according to the ARRIVE guidelines and with the institutional ethical approval. Briefly, the experimental procedures described in "Methods" were approved by the institutional animal care and use committee of Tohoku Medical and Pharmaceutical University based on the National Institutes of Health guide for the care and use of laboratory animals.

Consent for publication

Not applicable.

Competing interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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