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Design and Fabrication of Cubic Eggshell Containing Chick Embryo for a Novel Biomedical Platform*

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Abstract— **We propose a novel biomedical platform of a cubic (artificial) eggshell containing a chick embryo. The use of this proposed platform can eliminate the use of large animals as experimental models, making it more ethically accepted. It also has the advantages of low cost and a small size. The specific design and fabrication method of the cubic eggshell uses polydimethylsiloxane (PDMS) and polycarbonate, which were shown to achieve the required specifications of the proposed platform. The viability of the chick embryo in the cubic eggshell was confirmed through basic experiments. Finally, surgical tools were inserted into the cubic shell and the internal organs were operated on to confirm the use of the developed cubic shell as an appropriate platform for microsurgical training.**

I. Introduction

Microsurgery for the brain, eye, and joints is now a well-established method in modern medicine [1–3]. In microsurgery, surgeons use small surgical tools and perform operations under a microscope. For instance, tiny blood vessels can be connected and treated using micromanipulation under a magnified field of vision. The minimally invasive nature of this procedure contributes to a quick recovery time for patients. However, since a high level of technical skill is required for surgeons to handle tiny tissues and blood vessels, extensive surgical training is essential to improve the surgical performance of surgeons [4].

Even though surgical training using large animals such as pigs and dogs is known as a highly effective, the experimental procedures with large animals face ethical, practical and technical issues that limit their usage. Furthermore, as the ethical restrictions are becoming increasingly stringent, especially in Europe, scientists are small animals such as zebrafish or xenopus embryos in early developmental stages as alternatives to large animals in biomedical research [5–7]. The use of the embryo model for animal experiments and human risk assessment has increased dramatically, and the results obtained can help to predict the occurrence of diseases in vertebrates, including humans [8–10].

Among the embryo models, the chick embryo is a favorite alternative model used in experiments. For example, the chick embryo has been used extensively as a model system for studying angiogenesis since the development of the chick embryo is accompanied with the formation of new blood vessels [11]. It is known that chick embryos possess two extra-embryonic circulatory systems: the vitelline circulation and the chorioallantoic circulation on the chick chorioallantoic membrane (CAM) [12]. The CAM is a vascular membrane, that is pressed against the inside of the eggshell to absorb oxygen passing through the pores of the eggshell as the embryo grows [13][14]. The advantages of using chick embryos over large animal models are as follows:

1) Ethical acceptability: The ethical threshold of the usage of cultured chick embryos is lower than that of the application of other experimental animals [15]. The CAM is a functional vascular system, and the nerves do not develop before embryonic day 10 (the embryonic neural and immune systems do not develop until the second half of the normal chick development period) [16]. Therefore, human cells and tissues can be transplanted on the surface of the CAM without inflicting any pain on the embryo.

2) Low cost: Chicken eggs can be bought for less than 1 USD /egg from the local market. In addition, because the egg yolk acts as the source of nutrients, any culture medium is not required for the growth of chick embryo until hatching. This is one of the unique characteristic of the chick embryo.

3) High degree of usability: The chick embryo has a short life cycle that provides rapid experimental results [17], and the embryonic development can be easily observed by simply making a small hole in the eggshell. In addition, the antibacterial properties of the egg eliminate the need for a sterilized environment [18].

Against this background, we propose that chick embryos (in the egg) can be used as an alternative model for surgical training of surgeons for microsurgery, as shown in Fig. 1. In this method, the vascular network of the chick embryo is formed and distributed in an egg shell. The surgical instruments are then introduced into the egg, allowing surgeons to practice surgical procedures on the embryos with the vascular network. Then, by measuring the movement and operation time using the surgical tool, the surgical skills can be evaluated on a desktop environment, thus the eliminating the need for special facilities such as an operation room. However, a disadvantage of this model is the low observability due to the presence of the eggshell. We, therefore, propose that a new type of artificial eggshell should be used to achieve both high-observability and high-operability that is required for the surgical training for microsurgery. The design, fabrication, performance, and availability of the proposed artificial eggshell are discussed in this paper.

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II. Related Works

As mentioned above, a major problem of the use of chick embryo as a research model is that the embryo enclosed by the eggshell is not directly observable. To overcome this limitation, several groups have attempted to develop artificial eggshells. Datar et al. developed a simple model of shell-less chick embryo culture using a transparent glass bowl and a cover [19]. We would like to note that chick embryo should be moved to the artificial eggshell within two days from the start of the incubation (before formation of the blood capillary network) to prevent damage from the cracking eggshell. Kamihira et al. and Sobajima et al. used recipient eggshells (chicken or duck eggshell) as surrogates to culture quail or chicken embryos, and were able to successfully culture a high percentage of the embryos (43%) until hatching [20][21]. These researchers transferred embryos containing yolk and albumen to the recipient eggshell, sealed it with plastic thin film, and cultured it until hatching. Even though these methods realized the well observation of embryo development by the high-transparent shell, manipulation of chick embryos was difficult because the shell was composed of the rigid glass (high stiffness) and the fragile film (low stiffness). As a platform for tissue and blood vessel manipulation, artificial eggshell should have not only transparency but also appropriate stiffness.

To the best of our knowledge, there have been no studies on an artificial eggshell in order to realize high-observability and high-operability at the same time.

III. Design and Fabrication of Artificial Eggshell

A. *Required Specifications:*

The ideal artificial eggshell should have the following properties:

i) Culturing: high oxygen permeability and biocompatibility for the material of the shell are highly required. The chicken eggshell has a porous structure [22][23] that allows oxygen to pass in through it. Therefore, oxygen is critical to the growth of the vascular system and the development of the chick embryo, because cells have a limited stores compared with metabolic substrates such as glucose and amino acids [24].

ii) Observation and operation: As discussed earlier, transparency and appropriate stiffness of the artificial eggshell are required to achieve both high-observability and high-operability.

B. *Design of Artificial Eggshell:*

Based on the required specifications, the basic concept and the proposed design of artificial (cubic) eggshell are shown in Fig. 2. First, we consider a polyhedral shape (Fig. 2(a)) composed of a thin membrane with high oxygen permeability and transparency. In this case, the multi-dimensional structure makes it difficult and expensive to fabricate the polyhedron. On the other hand, a cubic shape (Fig. 2(b)) allows to ease of fabrication, and the posture of the embryo in the shell can be easily changed for ease of observation. However, when we use a thin membrane to obtain high oxygen permeability and transparency, it is

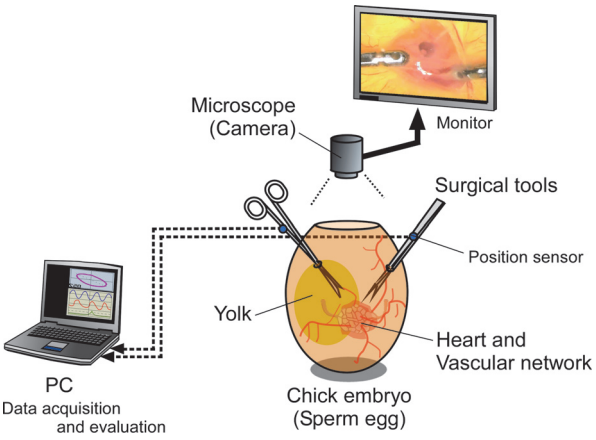

Figure 1. Conceptual image of proposed platform for surgical training using chick embryo with vascular network.

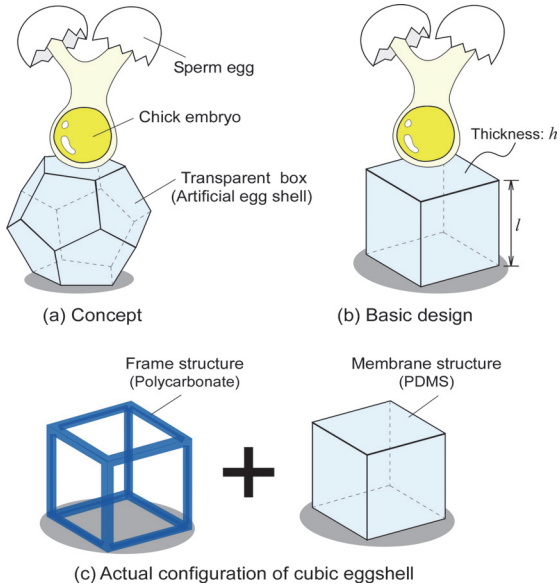

Figure 2. Basic design of the proposed artificial (cubic) eggshell.

difficult to make a self-standing structure due to the lack of structural stiffness. Therefore, we combine two different materials, and produced an optimal cubic eggshell as shown in Fig. 2(c). In this approach, the rigid frame structure helps to maintain the shape of the shell.

On the basis on this design, we determined that polycarbonate as a material of frame structure and polydimethylsiloxane (PDMS) as a material of membrane structure, respectively. Polycarbonate has high biocompatibility and stiffness (Young's modulus is approximately 2 GPa). PDMS has cross-linked network structures and is optically clear. It has been used in cell culture owing to its high biocompatibility. The mechanical strength and Young's modulus of the PDMS membrane depend on its thickness [25]. Most importantly, PDMS has high gas permeability [26]. Bo et al. investigated the permeability of PDMS membrane to oxygen and found that decreasing the membrane thickness resulted in a linear increase in permeability [27]. Therefore, as an artificial eggshell, if designed appropriately, PDMS membrane possesses not only enough stiffness and visibility but also good oxygen permeability.

The oxygen permeation rate influences the survival rate of the chick embryo inside the shell. The permeation coefficient of oxygen in PDMS membrane is expressed as [28]:

$$a = \frac{V_c h}{\tau S \Delta p} \tag{1}$$

where V_c is the total volume of oxygen (converted into the volume of oxygen in its standard state) permeating in a PDMS membrane with a thickness of h during a time period of τ, S is the effective oxygen-permeable area of the PDMS membrane; and Δp is the pressure difference converted from the difference in oxygen concentration between the two sides of the PDMS membrane. It has been reported that the permeation coefficient of oxygen, α, in PDMS membrane is approximately 3×10^{-11} $(cm^3cm)/(cm^2sPa)$ [29][30].

Based on this, we designed a cubic eggshell with a side length of l, using PDMS membranes with a thickness of h. A chicken egg takes 21 days to develop and hatch, and an embryo consumes approximately 6 L oxygen during this period. Therefore, we supposed that a total volume of 286 mL oxygen is required per day for the chick embryo. According to this calculation, we arrived at the values for pressure difference and time period: Δp = 101.33 kPa, and τ = 86400 sec, respectively. The egg volume is dependent on the species of chickens. It has been reported that the egg volume should be 40–60 cm^3 [31]. In this study, it was approximately 50 cm^3. Therefore, we designed the shell with a frame length of l = 40 mm. From the frame size, we calculated the total area of the PDMS membrane, and S = 9,600 mm^2. Therefore, according to equation (1), the thickness of PDMS membrane required for sufficient oxygen permeation for the normal development of a chick embryo, should be:

$$h = a\frac{\tau S \Delta p}{V_c} < 0.88 \text{ mm}. \tag{2}$$

According to equation (1), the thinner PDMS membrane is, the easier oxygen passes through the membrane, which is good for embryonic development. Therefore, a membrane with the least thickness should be used. On the other hand, since the Young's modulus of PDMS membrane is low (several hundred kilopascals), the shell cannot independently support the weight of the egg contents and maintain its shape. Hence, an inner frame with sufficient strength and high rigidity is required.

The thickness of the frame structure was designed to be 4 mm for the cubic structure to be suffiently strong. This means that there is a 40% reduction in the effective oxygen-permeable area of the PDMS membrane. Taking into consideration the decrease of the oxygen-permeable area, the final thickness of the PDMS membrane should be:

$$h < 0.55 \text{ mm}. \tag{3}$$

Fig. 3 shows the relationship between the thickness of the PDMS membrane and the amount of oxygen required to culture a chick embryo. The estimated curve was calculated from equation (1) and the measured data was obtained from an oxygen permeation analyzer (Systech Instruments Inc.).

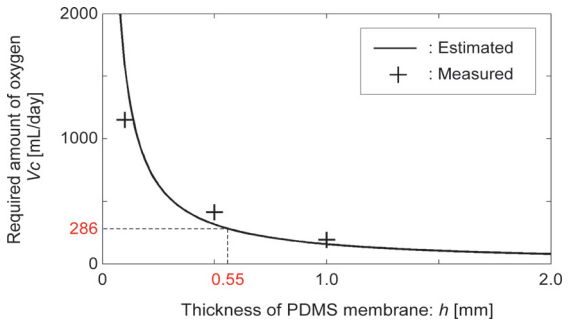

Figure 3. Relationship between the thickness of PDMS membrane and required amount of oxygen for chick embryo culturing.

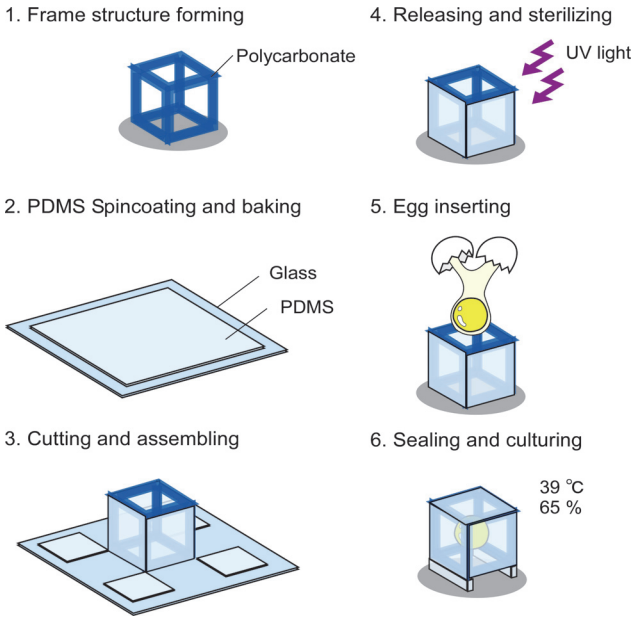

Figure 4. Fabrication process and culturing method for the cubic eggshell.

From this figure, we can estimate the how effective the thickness of PDMS membrane for the culturing. The effect of the thickness of PDMS membrane on the culture is discussed in Section IV.

C. *Fabrication Process:*

Fig. 4 shows the proposed fabrication of the proposed shell and methods for chick embryo culture.

1. A hollow cubic frame was manufactured from a block of polycarbonate by machining.

2. A thin PDMS membrane was fabricated as follows: PDMS and a curing agent (Sylgard 184, Dow Corning) were mixed well in a ratio of 10:1 and degassed in a vacuum desiccator. PDMS was then spin-coated onto the glass substrate. The thickness of the membrane was adjusted by changing the rotation speed. Thereafter the membrane was cured at 75°C for 20 min.

3. The membrane was carefully detached from the glass substrate, and, the thickness was measured by a digital vernier caliper. The PDMS membranes were attached to the hollow frame using PDMS glue on all sides except one.

4. The shell was filled with distilled water to identify any leaks. The shell was then sterilized by ultraviolet (UV) light.

5. The contents of the egg were then transferred into the shell through the open side of the cubic eggshell. To avoid damage to the embryo's vascular network, the eggs were cultured two days in an incubator and then cracked. The contents were then inserted in to the eggshell.

6. A piece of PDMS membrane was attached to the unwrapped face of the shell using liquid PDMS as glue. The eggs were incubated at 39°C with a relative humidity of 65%.

An overview of the fabricated cubic eggshell with a chick embryo is shown in Fig. 5.

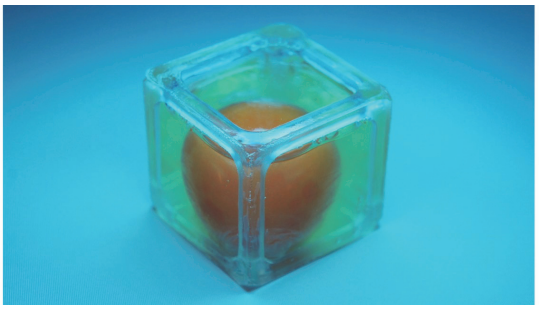

Figure 5. Fabricated cubic eggshell with a chick embryo.

IV. Experiments

A. *Ethics approval:*

The present study was approved by the Ethics Committee at the Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology. According to the Animals Act [15], a chick in an embryonic form is only a protected animal once half of the hatching period has elapsed [29]. Therefore, chick embryos before embryonic day 10 are not regarded as protected animals and experiments involving these embryos do not have to be registered.

During the experiment, we stopped the embryo culture based on the developmental stage and the embryonic days. On embryonic day seven (HH31), chick embryos were found to possess the following developmental properties [17][33]: 1) the circulatory system covers about 1/2 of the yolk, 2) egg-tooth is seen on tip of beak, 3) eyes are very conspicuous, 4) all organs are formed and the heart is completely inside the thoracic cavity, 5) the main blood vessels are more than 1 mm in thickness, and 6) the circulating blood volume of the chick embryo is approximately 260 mm^3. Furthermore, it has been reported that feather germs on the dorsal surface and thigh can be observed at HH31, and the body size of the developing chick is greater than 2 cm [34]. Therefore, we cultured chick embryos for up to seven days (HH31) so that there is not much feather germ formation but the developing chick is large enough to use as an animal model that allows tease of experimental manipulation.

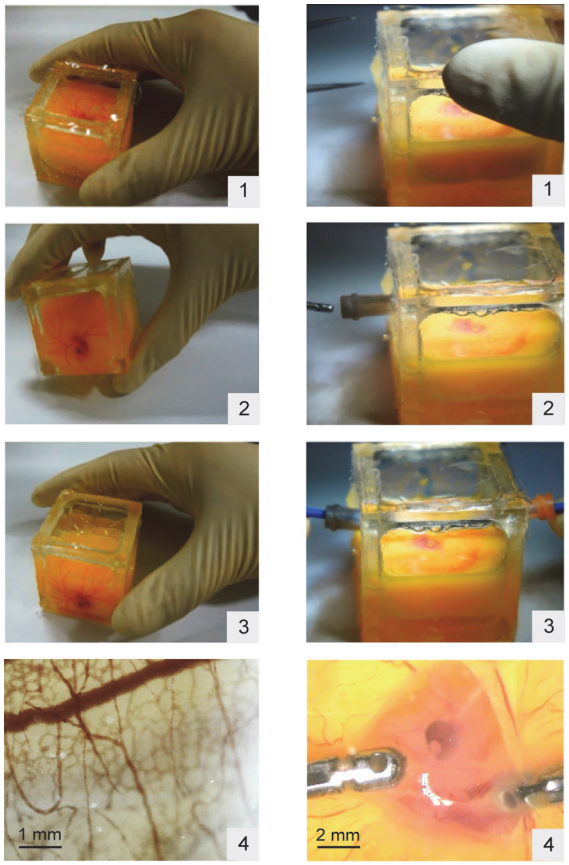

Figure 6. Overview of the demonstration for the observation and the manipulation of chick embryo using the developed cubic eggshell (see attached video).

B. *Demonstrations using the developed cubic eggshell:*

To confirm the usability of the developed shell, the demonstrations were conducted as shown in Fig. 6. In this experiment, the chick embryos were cultured for seven days in the shell (membrane thickness = 0.3 mm). As shown in Fig. 6(a1)–(a3), it was confirmed that by using the cubic shape of the shell, we can freely change the posture of the chick embryo to find the desired observation point. In addition, we confirmed the flow of cells in the formed blood vessels upon observation of the side wall of the shell (Fig. 6(a4)).

On the other hand, Fig. 6(b1)–(b4) shows an example of the mechanical manipulation of the chick embryo. In this experiment, holes were made by a tweezer. Then, the surgical tools (forceps) with a diameter of 2 mm were inserted into the inside of the shell. Finally, tissue and blood vessels of the chick embryo were manipulated under the microscope. Through this experiment, we confirmed that the mechanical strength of the PDMS membrane ensured that it remained during the manipulation. Therefore, the point at which tools were inserted did not crack, and there was no leaking of the egg contents.

C. *Viability of chick embryos in the cubic eggshell:*

To evaluate the validity of our design, the viability of chick embryos cultured in the developed eggshell was examined. We investigated the viability of chick embryos by counting the number of days that a heartbeat was confirmed. Fig. 7(a)

shows the result of the experiment. For comparison, we also cultured chick embryos in a plastic cup covered by a 10 μm-thick wrap (conventional method used as a control model), and in the cubic eggshell covered by 1 mm thick polystyrene plates (non-permeable model), and compared the survival rate of embryos in the three methods with that of embryos cultured in a natural eggshell. From this result, we found that the survival rate of embryos in natural eggshells was 100% during the culturing. Accordingly, we identified that the incubation conditions used (such as the temperature and humidity) were appropriate for chick embryo development. Conversely, the survival rate was approximately 90% during incubation using cubic eggshells with a 0.1 mm-thick membranes; this was greater than that observed with a 10 μm thick-film (67%). However, chick embryos died soon after we transferred the contents of eggs into the cubic eggshells in the non-permeable model. These results strongly suggested that oxygen permeation through the cubic eggshells was important for the survivability of chick embryos. Like thin plastic films, PDMS membrane is an excellent candidate for use in cubic eggshells.

Since the oxygen permeability is dependent on the thickness of the PDMS membrane, we also tested the effects of membrane thickness on the viability of chick embryos. As shown in Fig. 7(b), the survival rate of chick embryos using 0.3 mm-thick membranes was approximately 60%, which was lower than that observed for 0.1 mm-thick membranes. In addition, all the chick embryos cultured with 0.7 mm-thick membranes died before day seven. These results demonstrated that appropriate design of PDMS membrane thickness is crucial for oxygen permeation and, therefore, important for the development of chick embryos.

On the other hand, since the mechanical strength of the PDMS membrane is dependent on its thickness, the membrane strength should also be considered when developing this model. In this study, we found that chick embryos grew well in cubic eggshells fabricated with 0.1 mm- and 0.3 mm-thick PDMS membranes as estimated from equation (3). We also inserted surgical tools into the cubic eggshells fabricated with 0.3 mm-thick membranes and found no albumin leakage. These results indicate that our approach to design and fabricate an artificial cubic eggshell with 0.3 mm-thick membrane has proved to be an excellent candidate for a surgical training platform.

V. Discussions and Future Works

In our study, we found that the size of the formed blood vessels had grown to more than 1 mm by day 7–10, as shown in Fig. 8. After that, the blood vessels were covered by other internal organs as the body grows and were difficult to observe from the outside of the shell. This suggested that it is better to stop the culturing until day 10. We hypothesize the proposed cubic eggshell has huge potential for use as a microsurgical training platform, where the manipulation of 1 mm-thick blood vessels is required. In future work, we aim to compare chick embryo development between the normal egg and the proposed approach by dissecting the grown chick embryos in order to study any possible effects of the shape the shape and material of the shell on embryo development.

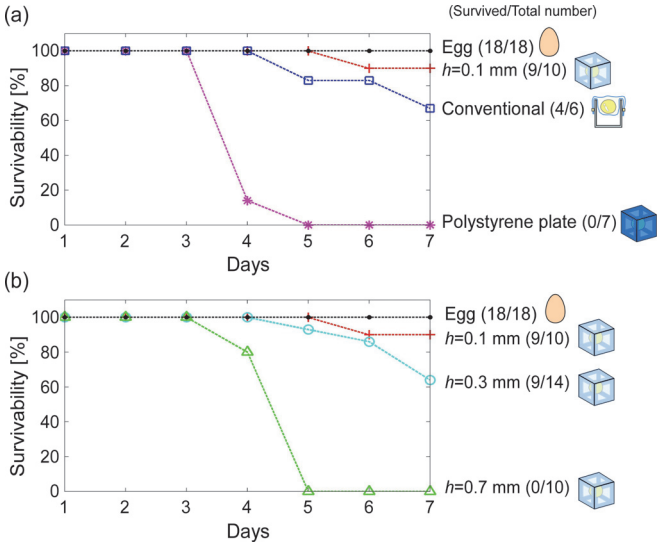

Figure 7. Survivability evaluation of chick embryos culturing in different conditions. (a) Comparison experimental result using the conventional and the proposed method. (b) Effect of the thickness difference (oxygen permeability) to the chick embryo survival rate.

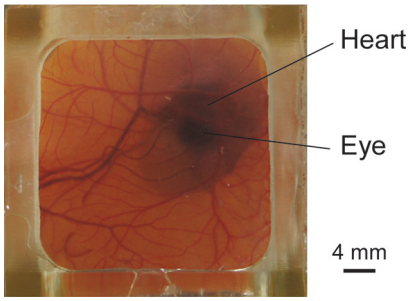

Figure 8. An overview of seven days cultivated chick embryo with 1 mm thick blood vessels.

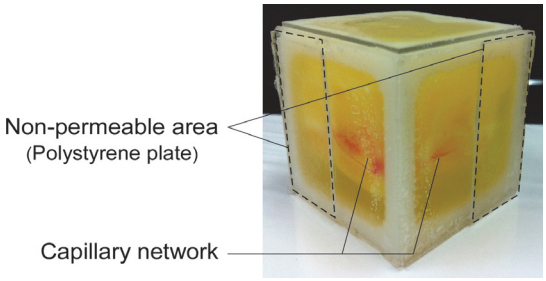

Figure 9. Inducing of capillary network by controlling the oxigen permeability of the cubic eggshell with the 3 days cultured chick embryo.

A training platform, as described in Fig. 1, will can be constructed and applied to microsurgery based on the achievements of conventional work [35]. In addition, microsurgical robots can be evaluated with fine accuracy [36] by using our platform to confirm their handling and suturing performance on tissues and blood vessels.

Aside from this, we found the behavior of the embryo during the experiment to be interesting. When we partially controlled the oxygen permeability rate of the shell by changing the thickness of the membrane, the direction of the developing capitally network was changed autonomously, as shown in Fig. 9. The capillary vessels attached to the sidewall of the shell in pursuit of oxygen required for their growth. This

is an added big advantage of our cubic eggshell model; blood vessel formations can be induced by designing the oxygen permeability pattern of the shell. Hence, we believe that our platform is useful, not only for surgical training, but also biomedical research such as in cardiovascular simulation, and drug testing, to name a few.

VI. Conclusions

In this paper, a chick embryo-based platform was proposed for use in microsurgical training. The cubic (artificial) eggshell was proposed as an alternative to overcome the problems associated with natural eggshell, such as observability and operability. The specific design and fabrication method for the cubic eggshell have been discussed in detail. The 7-day viability of the chick embryo in the shell, which was achieved by combining of a 4 mm-thick frame and a 0.1-mm thick membrane, was confirmed through basic experiments. Finally, we confirmed that surgical tools could be also inserted into the shell and used to perform microsurgical manipulations on the tissues of the chick embryo under a microscope. The use of the artificial cubic eggshell as a platform will help to create new methodologies for microsurgical training.

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