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--TITLE:

Ex Utero Culture of Mouse Embryos from Pregastrulation to Advanced Organogenesis

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SUMMARY:

An enhanced platform for whole-embryo culture allows continuous and robust *ex utero* development of postimplantation mouse embryos for up to six days, from pregastrulation stages until advanced organogenesis. In this protocol, we detail the standard procedure for successful embryo culture using static plates and rotating bottle systems.

ABSTRACT:

Postimplantation mammalian embryo culture methods have been generally inefficient and limited to brief periods after dissection out of the uterus. Platforms have been recently developed for highly robust and prolonged *ex utero* culture of mouse embryos from egg-cylinder stages until advanced organogenesis. These platforms enable appropriate and faithful development of pregastrulating embryos (E5.5) until the hind limb formation stage (E11). Late gastrulating embryos (E7.5) are grown in rotating bottles in these settings, while extended culture from pregastrulation stages (E5.5 or E6.5) requires a combination of static and rotating bottle cultures. In addition, sensitive regulation of O_2 and CO_2 concentration, gas pressure, glucose levels, and the use of a specific *ex utero* culture medium are critical for proper embryo development. Here, a detailed step-by-step protocol for extended *ex utero* mouse embryo culture is provided. The ability to grow normal mouse embryos *ex utero* from gastrulation to organogenesis represents a valuable tool for characterizing the effect of different experimental perturbations during embryonic development.

INTRODUCTION:

Intrauterine development of the mammalian embryo has limited the study of the early stages of postimplantation development[1,2]. The inaccessibility of the developing embryo hampers the understanding of key developmental processes occurring after the embryo implants into the uterus, such as the establishment of the animal body plan, specification of the germ layers, or the formation of tissues and organs. Moreover, the very small size of the early postimplanted embryo makes it difficult to observe by intravital imaging *in utero* before E10[3]. Due to the inability

to observe and manipulate living embryos at these stages, these limitations have restricted the study of early postimplantation embryogenesis to snapshots during development.

Protocols for *in vitro* culture of preimplantation mammalian embryos are well established, reliable, and regularly utilized[4]. Nevertheless, attempts to establish *ex utero* culture systems capable of supporting proper mammalian postimplantation embryo growth had limited success[5]. A variety of culture techniques have been proposed for over a century, mainly by culturing the embryos in conventional static plates[6–8] or rotating bottles (roller cultures)[5,9,10]. These platforms proved helpful in expanding the knowledge on mammalian development after implantation[11,12], despite being highly inefficient for normal embryo survival and limited to short periods. The embryos began to display developmental retardation and morphological anomalies as early as 24–48 h after culture initiation.

This study provides a detailed description for setting up the *ex utero* embryo culture system that allows continuous development from pregastrulation to advanced organogenesis stages over up to six days of postimplantation development[13]. This paper describes the improved roller culture protocol that supports the growth of E7.5 embryos (neural plate and headfold-stage) until the hind limb formation stage (~E11) and the extended culture from E5.5/E6.5 by combining culture on static plates and roller culture platforms.

PROTOCOL:

All animal experiments were performed according to the Animal Protection Guidelines of Weizmann Institute of Science and approved by relevant Weizmann Institute IACUC (#01390120-1, 01330120-2, 33520117-2). Healthy pregnant women were asked to give their informed consent to collect blood from their umbilical cord, as approved by the Rambam Medical Center Helsinki Committee (#RMB-0452-15). Healthy adults were asked for their informed consent to have blood collected according to the guidelines of the Weizmann Institute of Science Helsinki Committee (#1566-3).

1. Media preparation

1.1. Prepare the dissection medium using 500 mL of Dulbecco's Modified Eagle Medium (DMEM) without phenol red and L-glutamine, supplemented with 10% fetal bovine serum and filter-sterilized using a 0.22 µm filter.

NOTE: Keep the dissection medium at 4 °C for up to 1 month.

1.2. Prepare *ex utero* embryo culture medium (EUCM) freshly every culture day inside a biological hood using 25% of embryonic medium plus 50% rat serum and 25% human umbilical cord blood serum (HCS) or human adult blood serum (HBS).

1.3. To prepare embryonic medium, add 5 mL of glutamine, 5 mL of HEPES, and 5 mL of penicillin/streptomycin in 500 mL of DMEM without phenol red and L-glutamine. Prepare 10–15

mL aliquots and store them at 4 °C for up to two months.

NOTE: Double the antibiotic concentration if experiencing any contamination.

1.4. Heat-inactivate the rat serum at 56 °C for 30–45 min and filter through a 0.22 µm polyvinylidene difluoride filter. Use the serum for culture on the same day of inactivation. Thaw HCS or HBS and use it immediately for experiments.

NOTE: Commercial rat serum provides consistent results (a minimum of 4 lots were tested without evident variation). Alternatively, high-quality rat serum can be obtained *in-house* as described previously[8,14]. If HCS is not available, replace it with *in-house*-collected HBS. Rat serum, HCS, and HBS can be refrozen once and kept at -20 °C for use on a different day. Thaw and combine the refrozen serum with a larger volume of freshly thawed serum and do not refreeze it.

2. Collection of human umbilical cord blood serum and human adult blood serum

2.1. To isolate HCS, collect umbilical cord blood from healthy pregnant women on the day of the scheduled caesarian delivery.

2.1.1. Double-clamp the umbilical cord 5–7 cm from the umbilicus and transect between the clamps immediately after delivery of the infant.

2.1.2. Manually draw blood using a large-bore 14 G needle and a 50 mL syringe directly from the umbilical vein while the placenta remains *in situ* to avoid any traces of hemolysis.

NOTE: Collect blood after the infant is removed from the field of surgery, and umbilical blood has been drawn for clinical tests.

2.2 Dispense the collected blood to 5 mL procoagulant sterile test tubes and immediately transfer them to 4 °C for 15 min to allow complete coagulation. Centrifuge the coagulated test tubes at 2,500 × *g* for 10 min at 4 °C.

2.3. Discard tubes showing signs of hemolysis (pink-red serum). Collect the serum (yellow-color) using a 5 mL pipette and filter it through a 0.22 µm filter. Heat-inactivate the serum immediately in a 56 °C water bath for 45 min.

2.4. Prepare 1–1.5 mL aliquots of the inactivated serum and store them at -80 °C for up to six months. If needed, ship at -70 °C using dry ice.

2.5. For adult human blood serum collection, draw blood from healthy adults and immediately isolate serum following the same protocol described for umbilical cord blood serum. Store the HBS as heat-inactivated and filtered aliquots at -80 °C for up to six months.

NOTE: Human umbilical cord serum and adult human serum prepared freshly *in-house* gave superior results to commercially available serum. Collect HCS from healthy women, over the age of 18 and under 40 years scheduled for cesarian section delivery. Exclude women who gave vaginal birth and women with any chronic illness or active medical conditions, including gestational diabetes or hypertension.

3. *Ex utero* roller culture of embryos from E7.5 to E11

3.1. Setting up the roller culture incubator and gas regulator

3.1.1. Culture E7.5 or more advanced embryos using the roller culture incubator system. Turn on the rotator and the heating unit at 37 °C for at least 1 h before the embryo dissections. Add autoclaved water to the gas inlet bottle and the outlet test tube.

NOTE: Place a thermometer adjacent to the rotating drum and frequently check the stability of the temperature inside the incubator. Open the incubator as quickly as possible as prolonged opening time will increase the temperature and affect embryo development. When necessary, add more autoclaved water to the gas inlet bottle and the outlet test tube to keep them to a minimum of half capacity during culture. Protect the embryos inside the incubator from light by covering the incubator with a cloth.

3.1.2. Turn on the gas regulator module by pressing the main switch and subsequently turning on the Oxygen/Nitrogen and CO_2 controllers (**Figure 1A**). Set the **oxygen** and **CO_2** values to **5%** using the respective controllers. Open the gas regulator and set the **gas pressure** to **~6.5–7 pounds per square inch** (psi) by moving the voltage switch in the pressure transmitter (**Figure 1B**). Confirm the gas pressure value using a digital pressure gauge.

3.1.3. Monitor the gas flow by checking the rate of bubbles created inside the outlet water-filled test tube allocated inside the precision incubator. Set the proper bubble rate by closing/opening the gas flow valve on the lid of the water bottle. Ensure that the gas flow allows the formation of bubbles at a rate of **2–4 bubbles per second** or set the bubble flow at the first point where bubbling comes out to the water-filled outlet tube.

3.1.4. Fill the culture bottles with 2 mL of culture medium and plug them into the hollowed rotating drum for preequilibration for 1 h. Use the hollowed silicon bungs to seal the bottles to the drum. Keep sealed the empty spaces in the rotating drum using the solid bungs.

NOTE: The absence of bubbles in the outlet tube (no gas coming through the system) or an exceptionally high gas flow may affect embryo development. In the case of a lack of bubble flow to the outlet test tube, check that all silicon bungs are properly positioned in the rotating drum and sealing the system, and check that the water bottle is closed properly and all the tubing is connected correctly. A lack of bubbling coming into the water bottle may indicate a malfunction in the gas regulator.

3.2. Dissection of mouse embryos from pregnant mice

3.2.1. Preequilibrate the dissection medium inside an incubator at 37 °C and 5% CO_2 for a minimum of 1 h. Leave the lid slightly open to allow gas exchange.

3.2.2. Sacrifice the pregnant female by cervical dislocation, clean the abdomen of the female with 70% ethanol, and cut the skin and abdominal wall with scissors. Locate one end of the uterus and cut at the intersection between the ovary and the uterus. Next, cut along the uterus until the other end and transfer it to a 100 mm Petri dish filled with room temperature Dulbecco's phosphate-buffered saline (DPBS).

NOTE: Use of non-hormone-primed, naturally mated females is recommended.

3.2.3. Quickly wash the conceptuses in DPBS and cut in pairs to facilitate embryo handling. Move all the pairs of conceptuses to preequilibrated dissection medium in a 60 mm Petri dish and cut into individual conceptuses.

3.2.4. Remove the uterine wall of the conceptuses by tearing the uterine tissue using a pair of gross forceps. Use fine microsurgical forceps to cut the tip of the pear-shaped decidua. Insert the forceps next to the embryo parallel to its long axis, and open the forceps to split the decidua into halves.

3.2.5. Finally, leave the intact ectoplacental cone attached to the egg cylinder by grasping the embryo from the decidua and peeling the parietal yolk sac off the embryo using fine forceps.

NOTE: To avoid affecting the embryo, perform dissections on a microscope equipped with a heating plate at 37 °C within a maximum of 30–40 min. Dissect one litter of embryos at a time.

3.2.6. Immediately after dissection, transfer the embryos to a new plate filled with equilibrated dissection medium using a glass Pasteur pipette to prevent the embryos from sticking to the tissue debris.

3.2.7. Select those embryos in the neural plate/early head fold stage showing no damage in the epiblast and transfer 5–6 embryos per bottle to the preequilibrated glass culture bottles.

NOTE: To prepare the glass Pasteur pipette, cut the opening of the pipette to an adequate size to fit the embryos using a glass cutter, and keep it in ethanol to avoid contamination. Wash the glass pipette with PBS before transferring embryos. When transferring the embryos to the culture bottle, make sure to carry over as little volume of the dissection medium as possible to avoid diluting the EUCM.

3.2.8. Place the bottles in the rotating culture system at 37 °C, in an atmosphere of 5% O_2 and 5% CO_2.

3.2.9. Each day of culture, remove the bottles one by one from the incubator to evaluate embryo development under a stereomicroscope. Make sure to close the empty hole in the drum with a solid bung when removing a bottle. Cut the tip of a sterile plastic Pasteur pipette to fit the embryo size and move the embryos to a Petri dish to facilitate observation. Make sure that the embryos are always covered by medium.

NOTE: Minimize the time for which the embryos are outside the incubator to avoid detrimental effects in the embryos due to a decrease in body temperature. Handle the embryos carefully to avoid rupture of the yolk sac blood vessels, affecting embryo survival.

3.2.10. At culture day 1 (equivalent to E8.5), transfer groups of 3 embryos to a new bottle with 2 mL of fresh and preequilibrated EUCM containing extra 3 mg/mL of D-glucose and a gas mixture of 13% O_2, 5% CO_2.

3.2.11. After 48 h (equivalent to E9.5), transfer groups of two embryos to a new bottle with fresh preheated EUCM plus 3.5 mg/mL of glucose in a gas atmosphere of 18% O_2 and 5% CO_2.

3.2.12. At 72 h of culture (equivalent to E10.5), move each embryo to an individual bottle containing 1.5–2 mL of fresh medium supplemented with 4 mg/mL of glucose and a gas supply of 21% O_2 and 5% CO_2.

NOTE: Embryos reach their maximum growth about midnight of culture day 4.

3.2.13. Use fine forceps to remove the yolk sac and amnion, and detach the umbilical cord for proper observation of embryo morphology. Turn off the gas regulation module and precision incubator.

NOTE: Preequilibrate the culture medium for 1 hour by incubation inside a glass bottle in the roller culture with an adequate gas atmosphere according to the embryo stage. Clean the culture bottles and all incubator glassware after every use by performing three washes with running distilled water followed by an overnight wash submerged in 70% ethanol. Rewash three times with running distilled water, let the bottles dry overnight, and sterilize by autoclave. Likewise, autoclave the silicon plugs regularly. Thoroughly clean all dissection tools with 70% ethanol and dry-sterilize them.

4. Extended embryo culture from E5.5/E6.5 to E11

4.1. Culture pregastrulation (E5.5) and early gastrulation (E6.5) embryos until the early somite stage (E8.5) in static plates.

4.2. Preequilibrate the dissection medium for 1 h in an incubator with 5% CO_2 at 37 °C.

NOTE: To allow gas exchange, do not close the lid of the tube completely.

4.3. Prepare the culture plates by adding 250 µL of freshly prepared EUCM to each well. Place the plates inside a CO_2 incubator at 37 °C for preequilibration.

NOTE: Although 8-well plates are well-suited for embryo growth, culture can be done in any other plate by adjusting the volume of the medium according to the size of the plate.

4.4. Dissect egg cylinder embryos out of the uterus following the technique described for E7.5. Remove the parietal yolk sac of the embryo and leave the intact ectoplacental cone attached to the egg cylinder.

4.5. Transfer individual embryos into each well of the 8-well plate using a micropipette and place the plate inside the incubator with 5% CO_2 at 37 °C.

4.6. Image the embryos under a stereomicroscope and select for culture only those with a well-formed amniotic cavity, with no evident damage and without the Reichert's membrane.

NOTE: Use 20 µL pipette tips to transfer E5.5 embryos and 200 µL tips to transfer E6.5 embryos. In the case of cultures starting at E6.5, HCS can be replaced by freshly collected HBS.

4.7. Remove half of the medium and add 250 µL of freshly prepared prewarmed EUCM after 24 h of culture, ensuring that the embryos are always immersed in the culture medium. In the case of cultures starting at E5.5, after two days of culture (equivalent to E7.5), remove 200 µL and add 250 µL of new EUCM.

NOTE: During static culture, embryos might attach to the plate (mainly when using plastic plates), which will severely compromise embryo development. Attachment of the embryo to the plate is more frequent on the first day of culturing E5.5 embryos. To prevent attachment, carefully push the embryos away from the plate surface using fine, sterile forceps. Verify that only the ectoplacental cone remains attached to the surface of the plate.

4.8. Transfer the embryos into the roller culture at the early somite stage (4–7 somites; after three days of culture for embryos explanted at E5.5 and two days for cultures started at E6.5), following the same indications described previously for E8.5 stage embryos.

NOTE: Transferring the embryos to the roller culture at somite stages different than indicated above results in failure of further development. In contrast with E7.5 *ex utero* cultures, it is possible to maintain the embryos in a constant atmosphere of 21% oxygen and 5% CO_2, providing a slightly higher efficiency of embryo development than atmospheres with dynamic oxygen concentration.

REPRESENTATIVE RESULTS:

The roller culture conditions described for E7.5 embryos (late-gastrulation stage) support constant and normal embryo growth with an average efficiency close to 75% after 4 culture days (**Figure 2** and **Table 1**). The efficiency of embryo development may vary across diverse mouse

genetic backgrounds but is consistently robust (**Figure 2C**). Supplementation with HBS instead of HCS yields an efficiency of ~68% after 4 days of *ex utero* culture, depending on the genetic background of the mice (**Figure 2D** and **Table 2**). The embryos developed *ex utero* recapitulate proper development until approximately the 44-somite stage. Afterward, the embryos present embryonic abnormalities due to the absence of the allantoic placenta, resulting in insufficient oxygenation and nutrient supply given the increased body size at this stage.

Development of E6.5 embryos (early-streak) in static plates is correctly recapitulated with an efficiency of >90% until the early somite-stage E8.5, using EUCM with both HCS and HBS (**Figure 3**, **Table 3**, and **Table 4**) (see[8,13] for a detailed description of embryo staging between E5.5 to E8.5). *Ex utero* culture from gastrulation to advanced organogenesis by combining cultures on static plates followed by the roller culture in a constant 21% oxygen atmosphere gives an estimated efficiency of proper development of 55% and 26% to the 44-somite stage, using HCS and HBS, respectively (**Figure 3A**, **Table 3**, and **Table 4**). There is a delay of 1–2 somite pairs in these embryos compared to embryos developed *in utero*. The greatest drop in efficiency occurs at the transition from E8.5 to E9.5 due to failure of axial turning and closure of the neural tube.

Cultures starting from E5.5 pregastrulating embryos show efficiency of proper development to the early-somite stage (E8.5) of approximately 46%, and nearly 17% of the embryos will complete proper development after six days of culture after being transferred to the roller culture (**Figure 4** and **Table 5**). Extended *ex utero* culture prolongs the developmental delay in the embryos, with embryos explanted at E5.5 showing a delay of 2–4 pairs of somites compared to *in vivo* embryos. Nevertheless, morphogenesis and tissue development proceed properly until approximately the 42-somite stage.

The most common defects seen in the embryos for cultures initiated from E7.5, E6.5, and E5.5 are exemplified in **Figure 5A–C**. At the time of dissection, embryos with even minor damage to the epiblast or the extraembryonic region, as well as embryos retaining the Reichert's membrane, should be discarded. Likewise, early embryos will not grow properly (see **Figure 5B** for dead embryos) or display severe developmental delays (see **Figure 5B** for a delayed embryo). Attachment of the embryonic epiblast to the surface of the plate will affect development depending on the position and grade of attachment. Attachment of a part of the epiblast or the whole embryo will cause the failure of further development (see **Figure 5B** for an attached embryo).

The main abnormalities observed in the percentage of defective embryos at E8.5 (early somite stage) are the development of the posterior region outside the yolk sac or defects in the growth of the neural folds (**Figure 5A,B**). In the case of cultures started at E5.5, a frequently observed developmental defect is the presence of a small, underdeveloped epiblast (**Figure 5C**). At the time equivalent to E9.5, defects in the closure of the neural folds, failure of axial turning, or a deficiency in brain growth represent the most commonly observed abnormalities (**Figure 5**). The most frequently observed developmental defects at E10.5/E11 are anomalies in the head region, disruption of normal blood circulation in the yolk sac, and pericardial effusion (**Figure 5**). Rupture of one main blood vessel and blood outflow may cause subsequent death of the embryo. Notably,

proper growth of the embryo itself might be reached even in the absence of evident yolk sac circulation. Embryos kept in culture beyond the stage equivalent to E11 exhibit body shrinkage and death after few hours due to a lack of proper tissue oxygenation.

FIGURE AND TABLE LEGENDS:

Figure 1: Gas and pressure regulation system adapted to a roller culture incubator. (**A**) Top view of the gas regulation module connected to the roller culture incubator. N_2 and CO_2 enter the gas regulator to allow precise control of the oxygen/CO_2 concentrations and gas pressure. The gases are directed towards the mixing box, in which they are mixed by a centrifugal blower and injected into the incubator by a pump that generates positive pressure. The gas flows through the inlet into a water bottle and later to the sealed bottles. (**B**) Internal configuration of the electronic module for gas and pressure regulation. The voltage value set on the pressure transmitter regulates the pressure generated by the pump inside the gas mixing box (5–6 V to attain pressure of 6–7 psi in this specific model).

Figure 2: *Ex utero* culture platform supports growth of E7.5 embryos until advanced organogenesis. (**A**) Diagram depicting the E7.5 *ex utero* embryo culture protocol. (**B**) Representative bright-field images of groups of cultured embryos developing *ex utero* over 4 days, from late gastrulation (E7.5) to the 44-somite stage (E11). The typical variation in somite number assessed every 24 h is indicated. Scale bars = 500 µm. (**C, D**) Percentage of normally developed embryos at 1–4 days of culture starting from E7.5 divided by mouse parental strains and serum supplementation (**C**, human umbilical cord blood serum; **D**, human adult blood serum). Panel **A** has been modified from [13]. Abbreviations: EUCM = *ex utero* embryo culture medium; HCS = human umbilical cord blood serum; HBS = human adult blood serum.

Figure 3. Extended *ex utero* culture protocol for growing E6.5 early-gastrulating mouse embryos until late organogenesis. (**A**) Schematic illustration of the extended *ex utero* culture protocol combining static plates and rotating bottles systems. (**B**) Bright-field images of embryos cultured *ex utero* for five days from E6.5 to the 44-somite stage. The typical variation in somite number assessed every 24 h is indicated. Scale bars = 500 µm.

Figure 4: Continuous *ex utero* culture of pregastrulation mouse embryos from E5.5 until late organogenesis stages. (**A**) Schematic depiction of the *ex utero* culture the protocol for E5.5 embryos. (**B**) Representative bright-field images of embryos cultured *ex utero* for six days from E5.5 until the 42-somite stage. The typical variation in somite number assessed every 24 h is indicated. Scale bars = 500 µm.

Figure 5: Representative developmental defects observed in embryos cultured *ex utero*. (**A–C**) Bright-field microscopy images of abnormal mouse embryos grown *ex utero* starting from E7.5 (**A**), E6.5 (**B**), or E5.5 (**C**). A general description of the defect is provided on each image. Scale bars = 500 µm.

Table 1: Efficiency of proper development of embryos isolated at E7.5 days post coitum. The embryos were cultured *ex utero* for 4 days in EUCM (25% Human Umbilical Cord Blood Serum).

[-] indicates cultures not continued due to experimental requirements.

Table 2: Efficiency of proper development of embryos isolated at E7.5, cultured *ex utero* for 4 days, replacing Human Umbilical Cord Blood Serum with freshly isolated Human Adult Blood Serum. [-] indicates cultures not continued due to experimental requirements.

Table 3: Efficiency of proper development of embryos (B6D2F1/ICR) isolated at E6.5 and cultured *ex utero* for 5 days using EUCM (25% Human Umbilical Cord Blood Serum). The ex utero culture was done in static culture for two days (21% O_2) followed by three days in rotating bottles at 21% O_2. [-] indicates cultures not continued due to experimental requirements. Abbreviation: NA = not acquired.

Table 4: Efficiency of proper development of embryos (B6D2F1/ICR) isolated at E6.5 and cultured *ex utero* for 5 days using EUCM (replacing Human Umbilical Cord Blood Serum with freshly isolated Human Adult Blood Serum). The embryos were developed in static culture for two days (21% O_2) followed by three days in rotating bottles at 21% O_2. [-] indicates cultures not continued due to experimental requirements.

Table 5: Efficiency of proper development of embryos (B6D2F1/ICR) isolated at E5.5 and cultured *ex utero* for 6 days using EUCM (25% Human Umbilical Cord Blood Serum). The embryos were developed in static culture for three days (21% O_2) followed by three days in rotating bottles at 21% O_2. [-] indicates cultures not continued due to experimental requirements.

DISCUSSION:

The culture protocol presented herein can sustain proper and continuous mouse embryo development *ex utero* for up to six days, from E5.5 to E11. Previously, embryos at these developmental stages could develop normally in culture only for short periods (up to 48 h)[15]. The coupling of the gas regulation module to the roller culture incubator for precise control of oxygen concentration and hyperbaric gas pressure is critical for the proper mouse embryo culture described herein. Increasing the gas pressure to 7 psi enhances oxygen diffusion, allowing embryo development up to E11 in an atmosphere of up to 21% O_2/5% CO_2, in contrast to the previously employed conditions of 95% O_2[16], which may be harmful to the embryo in the long term. Further, oxygen concentration is known to play a critical role in embryonic development, as early postimplantation embryogenesis takes place under hypoxic conditions[17]. Accordingly, the successful culture of late-gastrulating embryos requires an initial 5% O_2 atmosphere, with a dynamic increase in oxygen concentration as the embryo grows. Remarkably, culturing pre/early-gastrulating embryos in static culture at 5% O_2 drastically decreased the efficiency of proper embryo development compared to 21% O_2, and they could not develop further to E9.5. The latter might be explained by the slower rate of nutrient and oxygen diffusion through the embryonic tissues in the static culture compared to the culture in rotating bottles[1,10].

Furthermore, the high content of rat and human umbilical cord blood serum provides more consistent results for growing early postimplanted embryos than media supplemented with only

rat serum[13,18]. Importantly, the serum used for embryo culture should be prepared from freshly extracted blood. Although high-quality rat serum for whole-embryo culture is commercially available, human serum should be isolated in-house. Supplementation with human umbilical cord blood serum can be replaced by serum isolated from human adult blood, which is widely accessible and still provides consistent and efficient embryo growth.

Successful and efficient embryo development *ex utero* is also highly dependent on accurate embryo isolation. First, the dissection procedure should be performed in dissection medium warmed at 37 °C, and the dissected embryos should be transferred into the culture bottles/plates within 30 min. Second, precise embryo isolation from the decidua and removal of the Reichert's membrane without damaging the epiblast is key for obtaining high efficiency of embryo development. Third, only embryos at the adequate stage should be selected for culture, as early/delayed embryos will not grow properly.

Handling the embryos during transfer is another essential point during the *ex utero* culture, mainly after the development of the embryonic yolk sac. Embryos should be transferred carefully because damage to major yolk sac blood vessels could affect proper development. Generally, the longer the period of embryo culture, the lower the efficiency of normal embryo development, i.e., embryos explanted at E7.5 will develop with higher efficiency than those explanted at E6.5 or E5.5. Moreover, the presence of antibiotics in the medium is fundamental to prevent contamination if a dissection microscope allocated inside a biological hood is not available.

It cannot be ruled out that other platforms, pressure levels, or conditions might enable similar or enhanced outcomes to the results obtained with the present protocol. Further optimization of the conditions described in this study is needed to reach an efficiency of embryo development equal to that observed by intrauterine development. Moreover, future development of a defined serum-free medium could help define the specific metabolic and chemical requirements during mammalian embryo development and reduce batch-to-batch serum variability. The need for constant nutrient and gas mixing after E8.5 using the rotating bottles culture in the current settings limits the long-term imaging capabilities during organogenesis stages. Future development of microfluidics devices enabling gas and nutrient mixture in static culture coupled to microscopy setups could help overcome this challenge.

Embryos cultured *ex utero* can be experimentally manipulated and kept in culture up to advanced organogenesis stages outside the uterus. We previously demonstrated the ability to introduce diverse perturbations in developing embryos, such as genetic manipulation by electroporation or lentiviral infection, live imaging, cell grafting, and teratogenic studies[13]. Ultimately, this platform may help uncover cell fate specification and organ formation mechanisms in mammals by allowing real-time experimentation in living mouse embryos for up to six days of early postimplantation development.

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DISCLOSURES:

J.H.H is an advisor to Biological Industries Ltd and has submitted a patent application covering the roller and static culture conditions described herein (filed by J.H.H. and the Weizmann Institute of Science). Other authors declare no competing interests.

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Figure 1

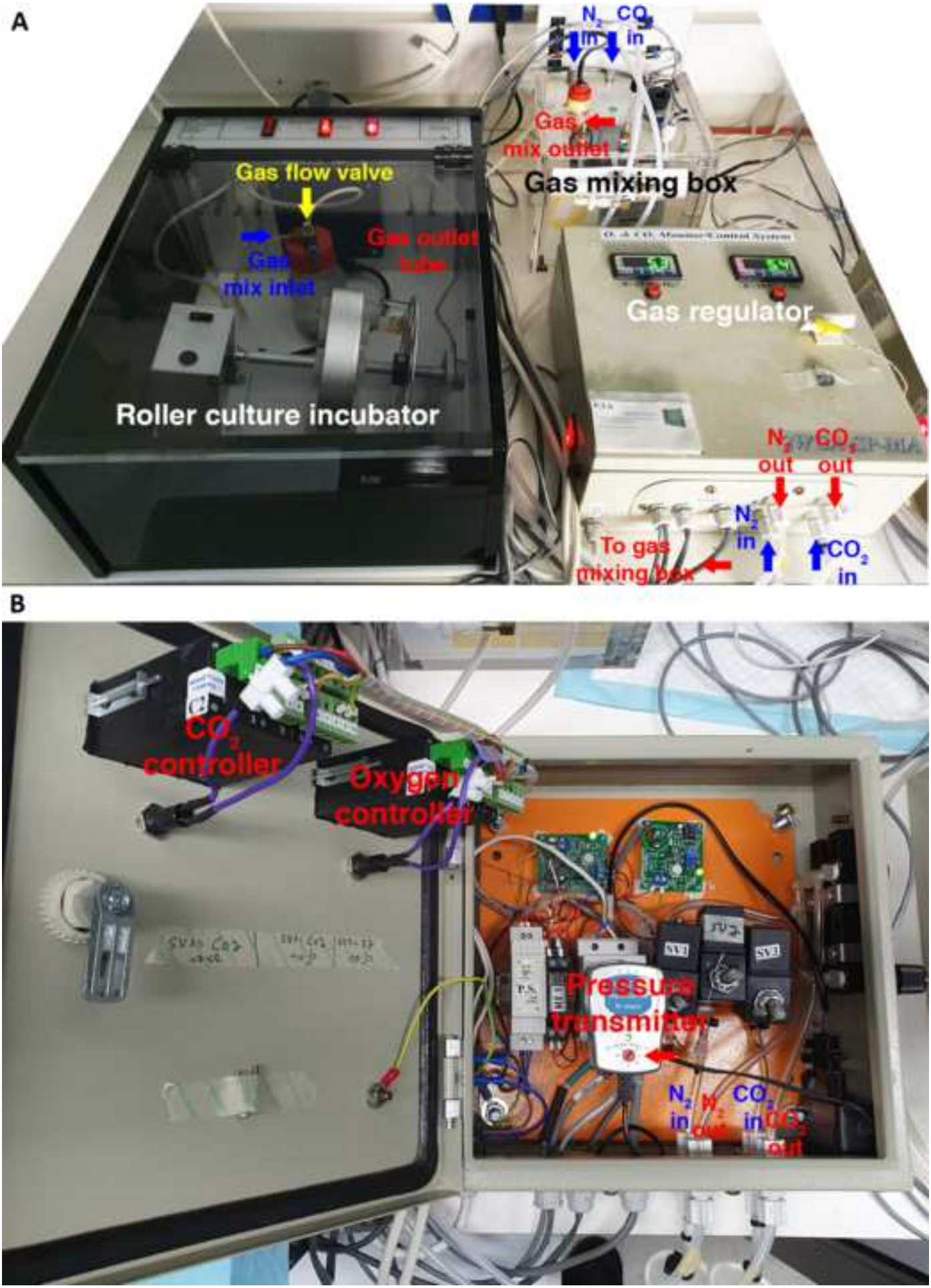

Figure 2

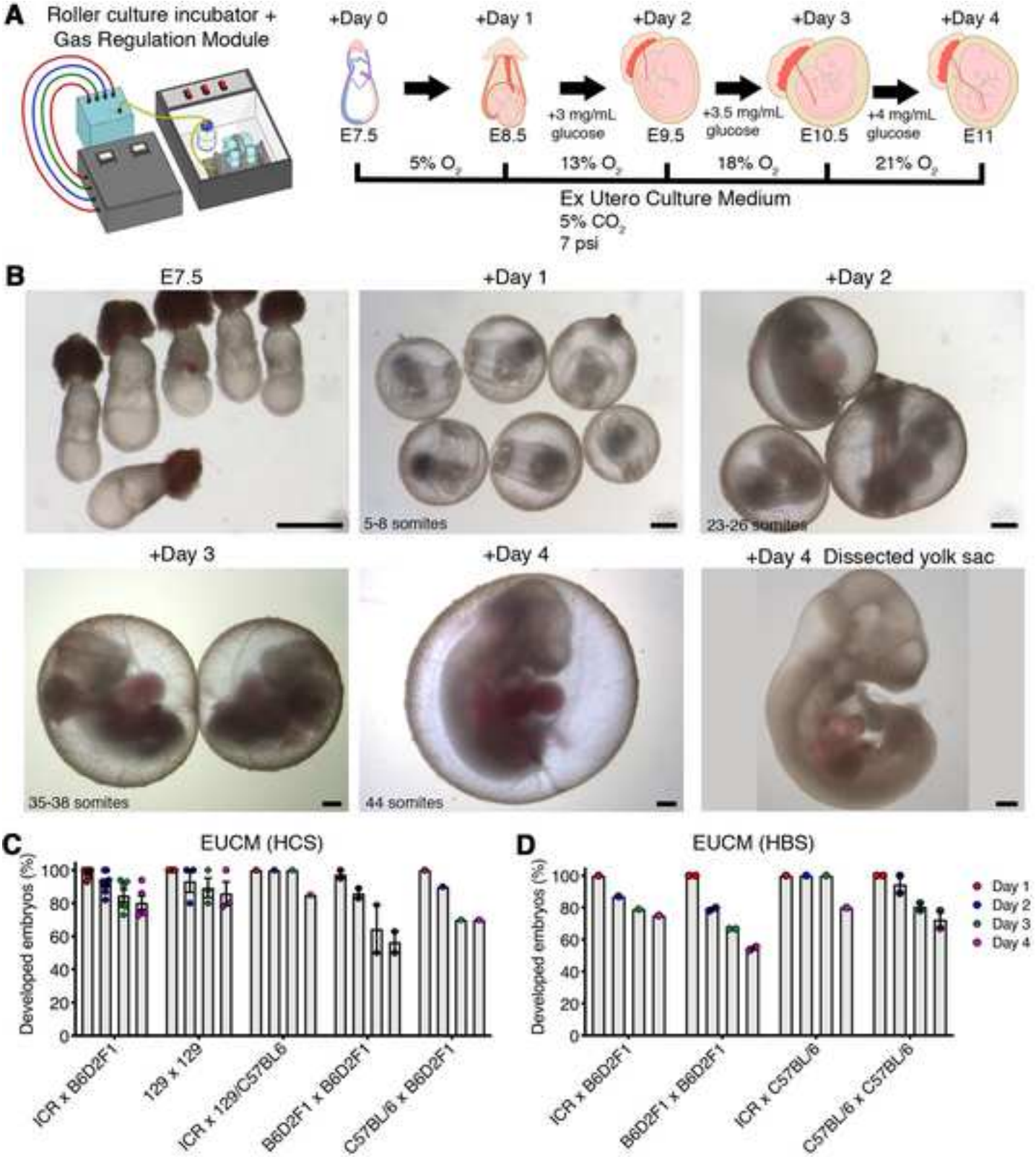

Figure 3

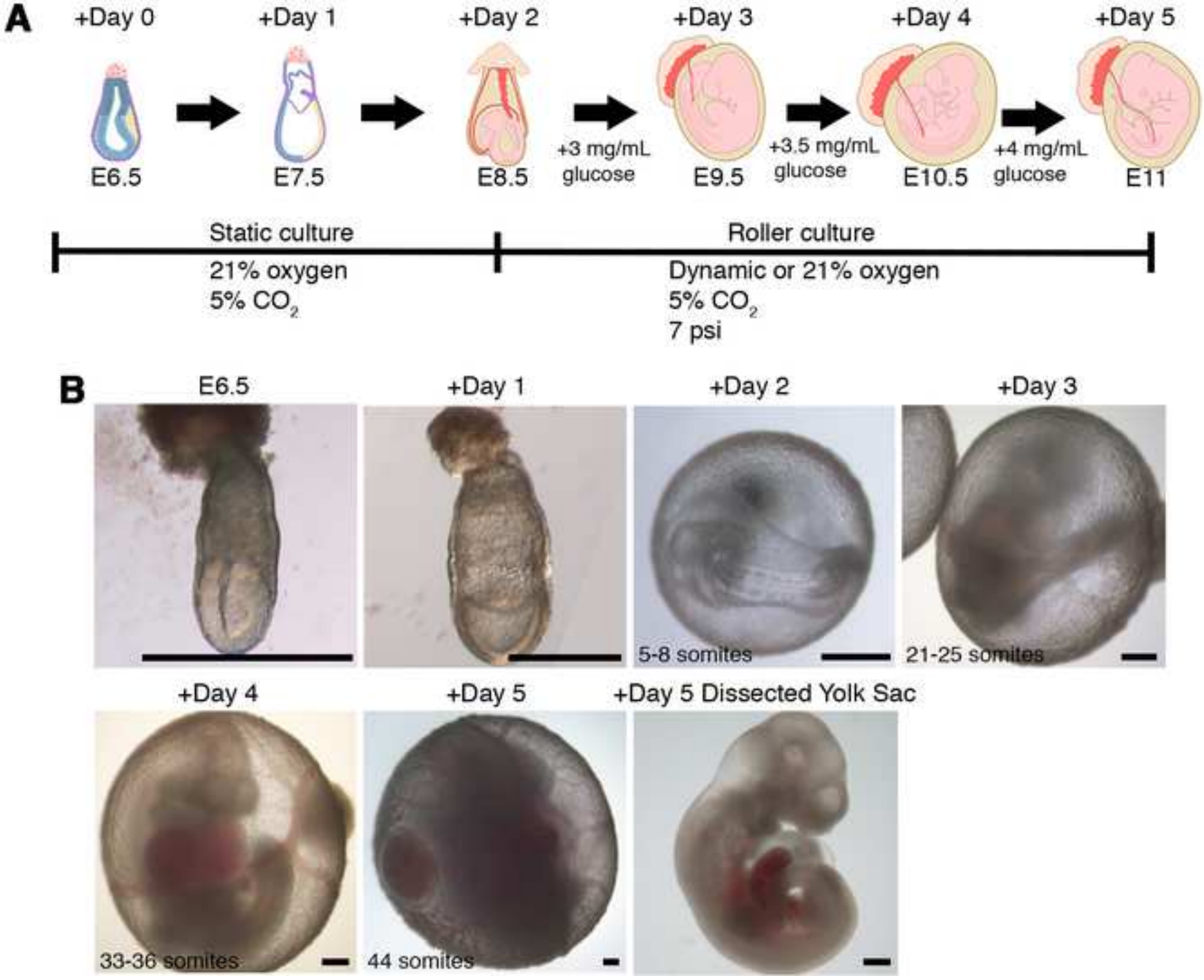

Figure 4

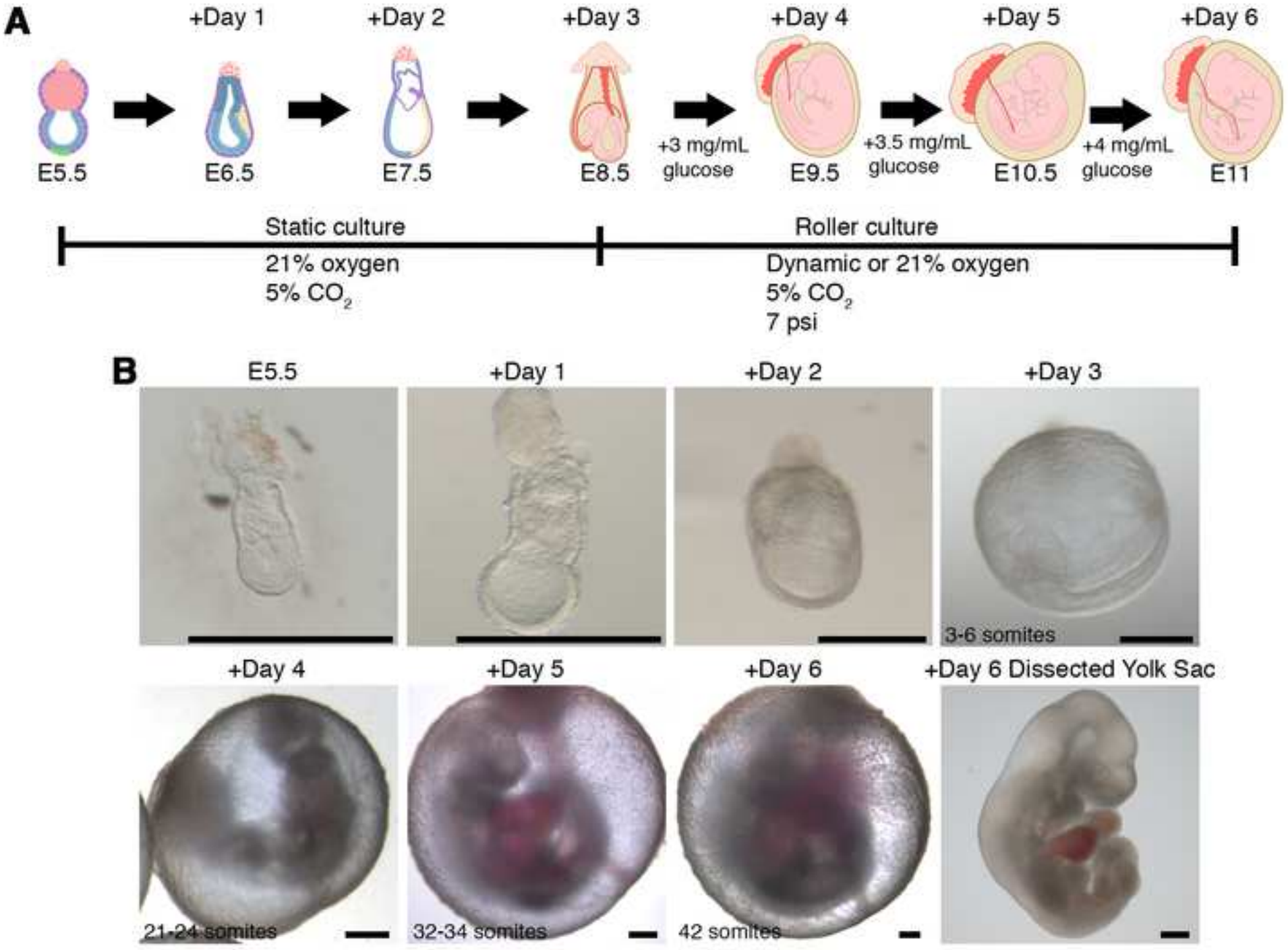

Figure 5

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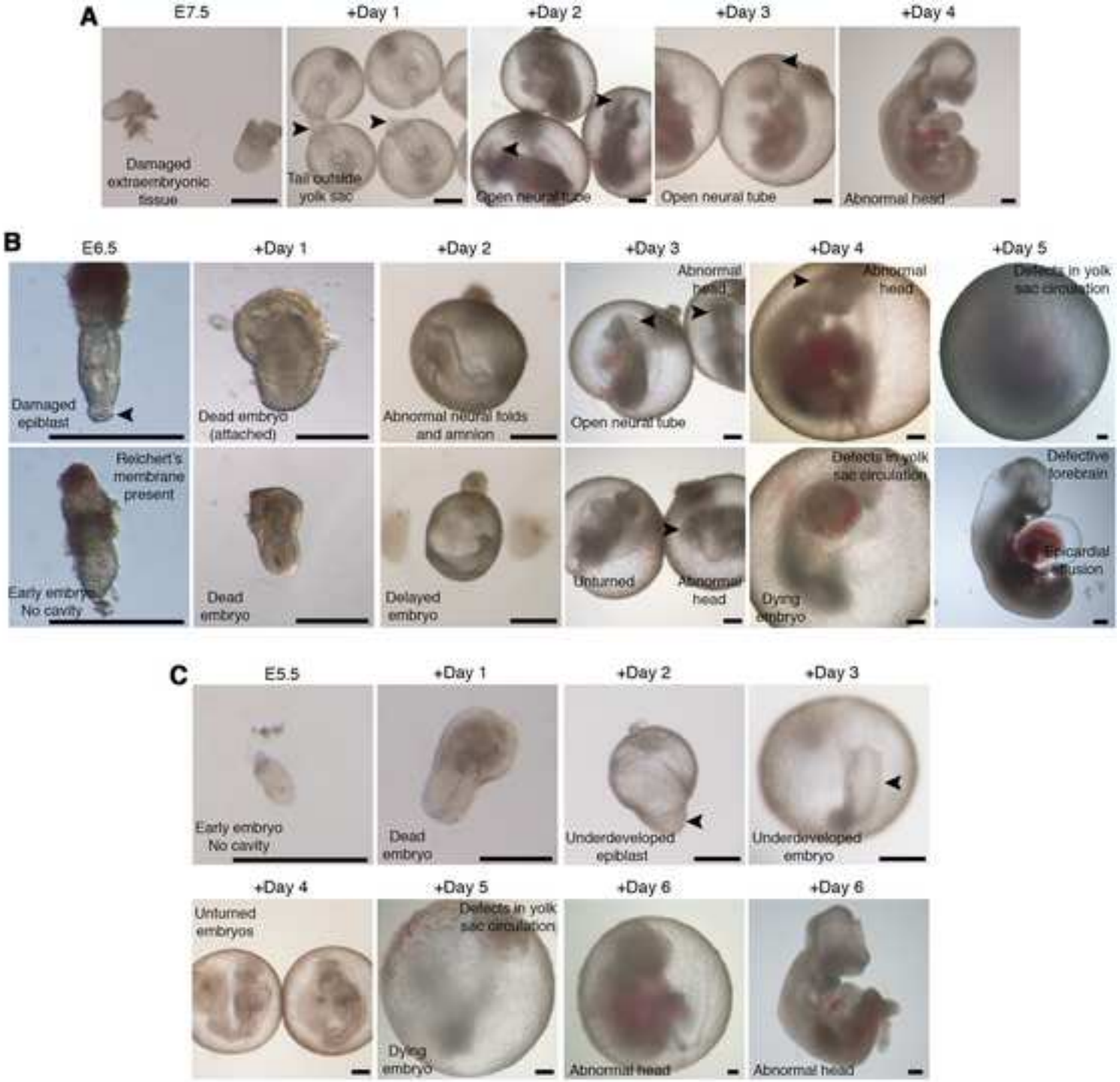

Table 1

Normally developed embryos	**+ Day 1 (E8.5)**	**+ Day 2 (E9.5)**	**+ Day 3 (E10.5)**	**+ Day 4 (E11)**
#1 ♀ICR × ♂B6D2F1	24/24 – 100%	21/24 – 87%	19/24 – 79%	18/24 – 75 %
#2 ♀ICR × ♂B6D2F1	23/23 – 100%	21/23 – 91%	18/23 – 78%	17/23 – 74%
#3 ♀ICR × ♂B6D2F1	11/11 – 100%	9/11 – 82%	8/11 – 73%	8/11 – 72.7%
#4 ♀ICR × ♂B6D2F1	10/10 – 100%	10/10 – 100%	-	-
#5 ♀ICR × ♂B6D2F1	35/35 – 100%	33/35 – 94%	-	-
#6 ♀ICR × ♂B6D2F1	21/22 – 95.5%	20/22 – 91%	20/22 – 91%	-
#7 ♀ICR × ♂B6D2F1	16/16 – 100%	16/16 – 100%	15/16 – 94%	15/16 – 94%
#8 ♀ICR × ♂B6D2F1	14/15 – 93%	14/15 – 93%	14/15 – 93%	13/15 – 86%
#9 ♀129 × ♂129	9/9 – 100%	9/9 – 100%	8/9 – 88%	7/9 – 78%
#10 ♀129 × ♂129	7/7 – 100%	7/7 – 100%	7/7 – 100%	7/7 – 100%
#11 ♀129 × ♂129	10/10 – 100%	8/10 –80%	8/10 – 80%	8/10 – 80%
#12 ♀ICR × ♂129/C57BL6 F2	13/13 – 100%	13/13 – 100%	13/13 – 100%	11/13 – 85%
#13 ♀B6D2F1 × ♂ B6D2F1	6/6 – 100%	5/6 – 83%	3/6 – 50%	3/6 – 50%
#14 ♀B6D2F1 × ♂ B6D2F1	18/19 – 95%	17/19 – 89%	15/19 – 79%	12/19 – 63%
#15 ♀C57BL6 × ♂ B6D2F1	10/10 – 100%	9/10 – 90%	7/10 – 70%	7/10 – 70%
Total	227/230 – 99%	212/230 – 92%	155/185 – 84%	126/163 – 77%

[-] Indicates cultures not continued due to the experimental requirements.

Table 2

Normally developed embryos	**+ Day 1 (E8.5)**	**+ Day 2 (E9.5)**	**+ Day 3 (E10.5)**	**+ Day 4 (E11)**
#1 ♀ICR × ♂B6D2F1	24/24 – 100%	21/24 – 87%	19/24 – 79%	18/24 – 75%
#2 ♀B6D2F1 × ♂B6D2F1	9/9 – 100%	7/9 – 78%	6/9 – 66.7%	5/9 – 55.5%
#3 ♀B6D2F1 × ♂B6D2F1	15/15 – 100%	12/15 – 80%	10/15 – 66.7%	8/15 – 53.3%
#4 ♀ICR × ♂C57BL6	10/10 – 100%	10/10 – 100%	10/10 – 100%	8/10 – 80%
#5 ♀ C57BL6 × ♂ C57BL6	6/6 – 100%	6/6 – 100%	5/6 – 83%	4/6 – 67%
#6 ♀ C57BL6 × ♂ C57BL6	9/9 – 100%	8/9 – 89%	7/9 –78%	7/9 –78%
Total	73/73– 100%	64/73 – 88%	57/73 – 78%	50/73 – 68.5%

[-] Indicates cultures not continued due to the experimental requirements.

Table 3 Click here to access/download;Table;Table 3 (4).xlsx

Normally developed embryos	+ Day 1	+ Day 2	+ Day 3	+ Day 4	+ Day 5
Experiment #1	37/38 – 97%	37/38 – 97%	5/7 – 71%	5/7 – 71%	3/7 – 43%
Experiment #2	28/28 – 100%	28/28 – 100%	6/8 – 75%	5/8 – 62.5%	4/8 – 50%
Experiment #3	28/28 – 100%	28/28 – 100%	11/13 – 85%	-	-
Experiment #4	29/30 – 97%	27/29 – 93%	15/15 – 100%	12/15 – 80%	8/15 – 53%
Experiment #5	8/8 – 100%	7/8 – 87.5%	-	-	-
Experiment #6	20/ 21 – 95%	NA	17/25 – 68%	-	-
Experiment #7	23/24 – 96%	23/24 – 96%	18/23 – 79%	15/23 – 65%	15/23 – 65%
Experiment #8	16/16 – 100%	16/16 – 100%	13/15 – 87%	13/15 – 87%	13/15 – 87%
Experiment #9	16/16 – 100%	16/16 – 100%	11/21 – 52%	9/21 – 43%	7/21 – 33%
Experiment #10	15/15 – 100%	15/15 – 100%	24/30 – 80%	17/30 – 57%	15/30 – 50%
Experiment #11	8/8 – 100%	8/8 – 100%	-	-	-
Experiment #12	7/7 – 100%	7/7 – 100%	-	-	-
Experiment #13	8/8 – 100%	8/8 – 100%	-	-	-
Experiment #14	13/13 – 100%	13/13 – 100%	-	-	-
Experiment #15	15/15 – 100%	15/15 – 100%	-	-	-
Experiment #16	31/32 – 97%	31/32 – 97%	-	-	-
Experiment #17	24/25 – 96%	24/25 – 96%	-	-	-
Experiment #18	23/23 – 100%	21/23 –91%	-	-	-
Experiment #19	15/15 – 100%	15/15 –95.5%	-	-	-
Experiment #20	20/21 – 99%	17/21 – 81%	-	-	-
Experiment #21	30/30 – 100%	30/30 – 100%	-	-	-
Total	414/421 – 98%	386/399 – 97%	119/157 – 76%	76/119 – 64%	65/119 – 55%

[-] Indicates cultures not continued due to the experimental requirements.

NA = Not acquired

Table 4 Click here to access/download;Table;Table 4 (2).xlsx

Experiment #1	10/10 – 100%	10/10 – 100%	7/10 – 70%	5/10 – 50%	4/10 – 40%
Experiment #2	8/8 – 100%	8/8 – 100%	3/8 – 37.5%	3/8 – 37.5%	2/8 – 25%
Experiment #3	16/16 – 100%	15/16 – 94%	9/16 – 56%	5/16 – 31%	4/16 – 25%
Experiment #4	8/8 – 100%	8/8 – 100%	-	-	-
Experiment #5	8/8 – 100%	7/8 – 87.5%	-	-	-
Experiment #6	13/13 – 100%	11/13 – 84.6%	-	-	-
Experiment #7	10/12 – 100%	9/12 – 75%	4/12 – 33%	3/12 – 25%	2/12 – 17%
Experiment #8	8/8 – 100%	8/8 – 100%	3/8 – 37.5%	-	-
Total	81/83 – 97.6%	76/83 – 91.6%	26/54 – 48%	16/46 – 34.8%	12/46 – 26%

[-] Indicates cultures not continued due to the experimental requirements.

Table 5

Normally developed embryos	+ Day 1	+ Day 2	+ Day 3	+ Day 4	+ Day 5	+ Day 6
Expt #1	5/5 – 100%	4/5 – 80%	4/5 – 80%	1/5 – 20%	1/5 – 20%	1/5 – 20%
Expt #2	5/9 – 56%	5/9 – 56%	5/9 – 56%	4/9 – 44%	3/9 – 33%	2/9 – 22%
Expt #3	8/10 – 80%	6/10 – 60%	6/10 – 60%	2/10 – 20%	2/10 – 20%	-
Expt #4	6/8 – 75%	5/8 – 62.5%	3/8 – 37.5%	1/8 – 12.5%	1/8 – 12.5%	1/8 – 12.5%
Expt #5	5/7 – 71%	6/7 – 43%	1/7 – 14%	1/7 – 14%	-	-
Expt #6	6/8 – 75%	6/8 – 75%	4/8 – 50%	2/8 – 25%	-	-
Expt #7	7/10 – 70%	6/10 – 60%	3/10 – 30%	1/10 – 10%	-	-
Expt #8	5/8 – 62.5%	4/8 – 50%	4/8 – 50%	4/8 – 50%	2/8 – 25%	1/8 – 12.5%
Total	47/65 – 72%	42/65 – 64.6%	30/65 – 46%	16/65 – 24.6%	9/40 – 22.5%	5/30 – 16.6%

[-] Indicates cultures not continued due to the experimental requirements.

We thank the editors and reviewers for the insights and constructive reviews that have significantly improved this manuscript.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use and use American English in all files.

Done

2. Please revise the following lines to avoid overlap with previously published work: 29-35, 43-44, 59-60, 63-68, 92-94, 101-103, 137-171, 177-179, 186-188, 190-198, 200-203, 206-207, 212, 222-223, 225-227, 240-243, 266-267, 269-272, 280-285, 286-293.
Changing the tense in the protocol to imperative will eliminate much of the overlap. Please refer to the attached iThenticate report.

We have reviewed the whole manuscript for overlap and changed all sentences to imperative.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (this includes the figures and the legends) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents, e.g., Glutamax etc

We confirmed that all products are mentioned in the Table of Materials and Reagents, and we do not use commercial language through the manuscript.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Any pronouns have been replaced through in the manuscript.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Key action steps should be protocol steps not notes. Please include all safety procedures and use of hoods, etc.

We have modified the whole text to ensure it is adjusted to the guidelines.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have checked the highlighted text and added more details according to the guidelines.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
8. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video. Avoid highlighting notes as these typically should not be action steps. Ensure that the highlighted steps are cohesive and represent the key message of your paper.
We simplified the protocol to contain 2-3 actions per step and confirmed that the manuscript fits to the proper spacing, margins and font.

9. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.
Done.

10. Please add all items (plastic and glassware, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.
Done.

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12. IF embryos were euthanized later, please indicate the method without highlighting it.
We indicate the cause of embryo death in the lines 239-241 of the manuscript.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This protocol describes critical adaptations to existing methodologies in order to successfully extend the range of whole embryo culture. This provides a window into embryonic developmental events that occur from pre-gastrulation to mid-gestation by using a combination of static and rotating culture. The use of human umbilical cord blood serum and human adult blood serum improves culture media that has previously relied on high quality rat serum alone. Overall this is a protocol that is of interest to developmental

biologists who would like to manipulate embryos ex utero.

Major Concerns:
There are no major concerns.

Minor Concerns:
1. Commercially available rat serum for whole embryo culture is expensive and the cost of shipping alone can be prohibitive depending on your location. There exists a protocol for preparation of high quality rat serum (Takahashi 2014) that could be referenced for those that would like to make their own. Rivera-Pérez 2010 also described this and provides detailed descriptions of morphological landmarks that could be very useful for those who are less familiar with pre-somitic stages.
Response: We thank the reviewer for the constructive comments. We now included a note referencing both papers describing the in-house isolation of rat serum (lines 97 to 99). Further, in the results section from gastrulation to somitogenesis we now reference the work by Rivera-Perez to direct the reader to their detailed descriptions of these embryonic stages.

2. While the tables are informative, it would be easier to grasp the global picture if the data were represented in graphs that show the variability by condition (strain or serum for example). Similarly, if the authors have details about the batches of serum, it would helpful to plot that as well in order to have an idea of the batch-to-batch variability that may be encountered (which can be problematic even when using commercially produced sera).
In addition to the tables, we include in the current version of Figure 2 (panel C and D) the data represented in graphs divided by mouse parental strain, both for media supplemented with human cord blood serum or with adult blood serum. As a note, we also include a sentence stating that the results were obtained with at least 4 different serum lots, without noticeable differences between them.

Reviewer #2:
Manuscript Summary:
In this article by Aguilera-Castrejon A et al,. a novel culture protocol that allows ex-utero culture of mouse embryos for up to 6 days post implantation is described. It covers pre-gastrulation stage E5.5 to the post-implantation stage of development, which corresponds to the hindlimb formation stage at E11. This very spectacular technical breakthrough is achieved by combining two different culture methods, a classical static culture-based one for embryos from the pre-gastrulation stage E5.5 to the late-gastrulation stage E7.5 and a rotary culture system for post-implantation stage embryos from E7.5 up to E11. Both those conditions require additionally a precise regulation of different factors including e.g. O2 and Co2 concentrations, atmospheric pressure, and different culture media composition. Moreover, proper handling of embryos during dissection seems to be also very important for the success of such a culture system, as stressed multiple time by the authors. Altogether, the novelty, complexity and uniqueness of the protocol described in this article makes it very relevant for publication in JoVE with some very minor modifications needed.

Major Concerns:
There are no major concerns for publication

Minor Concerns:
The culture setup involved in the ex-utero culture of mouse embryos seems very important and complex, as a consequence possible addition of a figure specifically dedicated to the technological/technical aspect of this culture setup and more detailed info than the current figure 1A could help the readers to understand better the various requirements needed to replicated this protocol.
We thank the reviewer for the positive comments on the manuscript. We now include a separate figure (Fig. 1) dedicated to describing the gas regulator configuration, how to operate it and set it for embryo culture.

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Author: Alejandro Aguilera-Castrejon et al

Publication: Nature

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