

Video Article

Ex vivo Culture of Mouse Embryonic Skin and Live-imaging of Melanoblast Migration

Richard L. Mort¹, Margaret Keighren¹, Leonard Hay¹, Ian J. Jackson¹

¹MRC Human Genetics Unit, MRC IGMM, Western General Hospital, University of Edinburgh

Correspondence to: Ian J. Jackson at Ian.Jackson@igmm.ed.ac.uk

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Abstract

Melanoblasts are the neural crest derived precursors of melanocytes; the cells responsible for producing the pigment in skin and hair. Melanoblasts migrate through the epidermis of the embryo where they subsequently colonize the developing hair follicles^{1,2}. Neural crest cell migration is extensively studied *in vitro* but *in vivo* methods are still not well developed, especially in mammalian systems. One alternative is to use *ex vivo* organotypic culture³⁻⁶. Culture of mouse embryonic skin requires the maintenance of an air-liquid interface (ALI) across the surface of the tissue^{3,6}. High resolution live-imaging of mouse embryonic skin has been hampered by the lack of a good method that not only maintains this ALI but also allows the culture to be inverted and therefore compatible with short working distance objective lenses and most confocal microscopes. This article describes recent improvements to a method that uses a gas permeable membrane to overcome these problems and allow high-resolution confocal imaging of embryonic skin in *ex vivo* culture⁶. By using a melanoblast specific Cre-recombinase expressing mouse line combined with the *R26YFP* reporter line we are able to fluorescently label the melanoblast population within these skin cultures. The technique allows live-imaging of melanoblasts and observation of their behavior and interactions with the tissue in which they develop. Representative results are included to demonstrate the capability to live-image 6 cultures in parallel.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51352/>

Introduction

Traditionally embryonic skin has been cultured by dissecting from the mouse embryo and mounting on a polycarbonate Nuclepore membrane. The membrane is then floated on culture medium, thus maintaining an air-liquid interface across the surface of the developing tissue^{3,4}. This technique has been used to assay melanoblast behavior by fixing the tissue and assessing melanoblast distribution using β -galactosidase as a marker⁷. We have developed a method that allows live-imaging of fluorescently labeled melanoblasts in *ex vivo* skin culture⁶. Here we describe the method in detail from dissection, to setup, to live confocal imaging and include some recent improvements.

Melanoblasts are the embryonic precursors of melanocytes, the cells that produce pigment in hair and skin. Melanoblasts arise in the neural crest adjacent to the neural tube at around embryonic day 9 (E9) in the developing mouse embryo. Subsequently they migrate along a dorsolateral pathway between the ectoderm and the developing somites. At E12.5 they move from the dermis to the epidermis where they proliferate and continue their migration. The primary hair follicle pattern begins to form in the epidermis at E14.5 and by E15.5 melanoblasts are localizing to these follicles. For a review of melanoblast/melanocyte development see Thomas & Erickson (2008)¹. In order to label the melanoblast population we have combined *Tyr::CreB* animals that express Cre-recombinase driven by the mouse tyrosinase promoter⁸ with *R26YFP* animals that express yellow fluorescent protein (YFP) conditionally from the *ROSA26* locus⁹.

We describe a method to culture embryonic skin and capture images using an inverted confocal microscope. It is adapted from the original method described in Mort *et al.* (2010)⁶. The present method allows imaging in a 6-well format and removes the reliance on Nuclepore membranes and on matrigel to support the culture. Instead using a small block of 1% agarose to stabilize the embryonic skin. Removing the reliance on matrigel is important especially in situations where the response of the embryonic skin to soluble growth factors is the focus of study. Our original method has already been used to gain new insights into melanoblast development¹⁰⁻¹³ and we anticipate that the improvements we describe here will make it more powerful as an experimental technique especially where multiple parallel cultures are a requirement.

Protocol

All animal work was performed in accordance with institutional guidelines under license by the UK Home Office (project license number PPL 60/3785 and 60/4424).

1. Preparation

1. Label the melanoblasts in the developing skin by combining an appropriate Cre-recombinase expressing mouse line with a fluorescent reporter mouse line. *Tyr::CreA*, *Tyr::CreB*⁸ and *Wnt1::Cre*¹⁴ have been used successfully as Cre-expressing lines and *R26YFP*⁹ as a reporter line.
2. Prepare the culture medium in a laminar flow hood; supplement Dulbecco's Modified Eagle Medium (DMEM) with (1% v/v Penicillin/Streptomycin), 10% v/v fetal calf serum and 0.584 gm/L L-glutamine.
3. The live imaging apparatus is outlined in **Figure 1** Sterilize the imaging chamber base by wiping down with 70% ethanol. Sterilize the inserts, imaging clips, and O-rings by soaking overnight in 70% ethanol. Use a sterile lid from a 6-well plate as the lid of the chamber.
4. The bottom surface of the imaging chamber consists of a lummox membrane held in place by an O-ring (**Figure 1**). Start with a 35 mm gas permeable lummox dish. Place the dish over the imaging chamber and cut out the gas permeable membrane with a single edged razor blade. Push the O-ring over the membrane to secure it to the imaging chamber.
5. The imaging clips (6 in total) clamp the embryonic skin in place (**Figure 1**) before use they must be filled with 1% agarose. Prepare a 2% solution of agarose with sterile dH₂O by microwaving and cooling to 60 °C. Mix 1:1 with 10 ml culture medium (see step 2) preheated to 60 °C. Stand the 6 imaging clips in a sterile 6-well plate. Drip the 1% agarose solution into the center of each clip using a fine pastette and allow to cool. Add 1 ml cold sterile PBS to each well to prevent the agarose from drying out. The imaging clips can be stored for several hours in PBS.
6. To carry out the delicate separation of embryonic skin and to handle the dissected tissue use the 'hair stick method' as described in Kashiwagi *et al.*³ Instead of using a chopstick a bamboo kebab skewer can be used. Make a small slit in the flat end of the skewer using a single edged razor blade and insert a soft toothbrush bristle. Trim the skewer so that it is approximately 15 cm in length.
7. Use a confocal microscope with a full environmental chamber or stage top chamber providing 5% CO₂ in air. Prewarm the microscope stage to 37 °C for at least 1 hr prior to imaging. This helps to prevent sample drift caused by expansion of the stage components as they warm up.
8. Prepare a multi-position set using the microscope's control software so that one can quickly and easily center on the middle of each culture to set up an experiment.

2. Procedure

1. Mate the mice and designate the first day post coitum as day E0.5. At day E14.5, cull the female by cervical dislocation and expose the body cavity by making a small midline incision, pulling apart the skin towards the head and tail and then cutting the underlying peritoneum and peeling back to either side of the body. Dissect the uterus into cold sterile PBS by holding firmly with forceps and snipping along the mesometrium using surgical scissors. Keep the uterus on ice until required. For a detailed description see Shea *et al.* (2007)¹⁵.
2. Dissect embryos from the uterus by first cutting the length of the uterine wall with surgical scissors to release the decidua and then dissecting the embryos from the decidua by pulling them apart with two pairs of fine forceps. Screen for fluorescent reporter expression (if necessary).
3. Cull embryos by decapitation prior to dissection. Subsequently remove the tail and hind limbs and then the forelimbs using a single edged razor blade. Retain tissue for genotyping if required.
4. Make an incision in the ventrum close to the midline in a rostro-caudal direction. Using the hair sticks described in step 1.6, carefully tease away the skin from the underlying connective tissue rotating the embryo at the same time. Do not remove the skin completely but rather leave it attached along the ventral edge furthest from the first incision.
5. It is important not to let the skin become screwed up as it is then very difficult to mount. To prevent this, flatten out the skin using the hair sticks (dermal side up) on the surface of a dry Petri dish and then cut away from the embryo trunk using a single edged razor blade. The dermal side can be distinguished from the epidermal side by careful observation of the junction between the dissected tissue and the embryo before the skin is removed. The area of the dissected tissue is approximately 0.5 mm² when dissecting an E14.5 embryo.
6. Place an imaging clip on top of the skin so that the agarose is touching the dermal side of the tissue. Allow to stand for 60 sec and then carefully tease the edges of the tissue up the sides of the clip. Invert the clip and use the hair sticks to flatten out the skin and remove any air bubbles, taking care to touch the surface of the tissue (in the area that will be imaged) as little as possible.
7. Use silk suture thread to tie the skin to the imaging clip using two simple thumb knots on either side of the imaging clip so that they tighten and pull the skin into the groove close to the top of the clip. Invert the clip, push inside the imaging insert and place in the base. Fill the insert with ~5 ml of culture medium (see step 1.2). **Figure 1** outlines the assembly of the imaging chamber.
8. Repeat Protocol for the remaining 5 samples.

3. Live Imaging

1. The imaging chamber has the same dimensions as a standard 6-well plate. Use an appropriate plate insert to mount the chamber on the stage of the confocal microscope.
2. An automated stage is required to image the cultures in parallel. Define the six positions to image in the confocal software. The exact settings will depend on the capability of the microscope used. Typically a small z-stack (3-5 z-positions to account for any stage drift) is used in each of the 6-positions and a z-stack is captured every 2 min for 18-24 hr. For live-imaging applications it is generally advantageous to use a wider than optically optimal pinhole setting to reduce laser power and the number of z-sections required.

Representative Results

Figure 2 shows representative results from a time lapse experiment using embryonic skin from *Tyr::CreB x R26YFP* embryos at E14.5. 6 embryonic skin cultures were imaged by confocal microscopy every 2 min for 18 hr. The freeware image analysis software package ImageJ was used to analyze the behavior of the YFP-labeled melanoblasts in the 6 movies. The melanoblasts were automatically tracked using the wrMTrack plugin developed by Jesper S ndergaard Pedersen (<http://www.phage.dk/plugins/wrmtrack.html>) the tracking results are displayed in **Figure 2**. **Movies 1-3** are representative of the experiment and correspond to panels A and G. They show the Transmitted light, YFP and composite views respectively. The migrating melanoblast population is extraordinarily active. Cells move almost constantly and only pause when they are about to

divide by mitosis. It is also possible to observe the development of the primary hair follicle pattern over the culture period in the transmitted light channel (**Figures 3A-3X** and **Movie 1**).

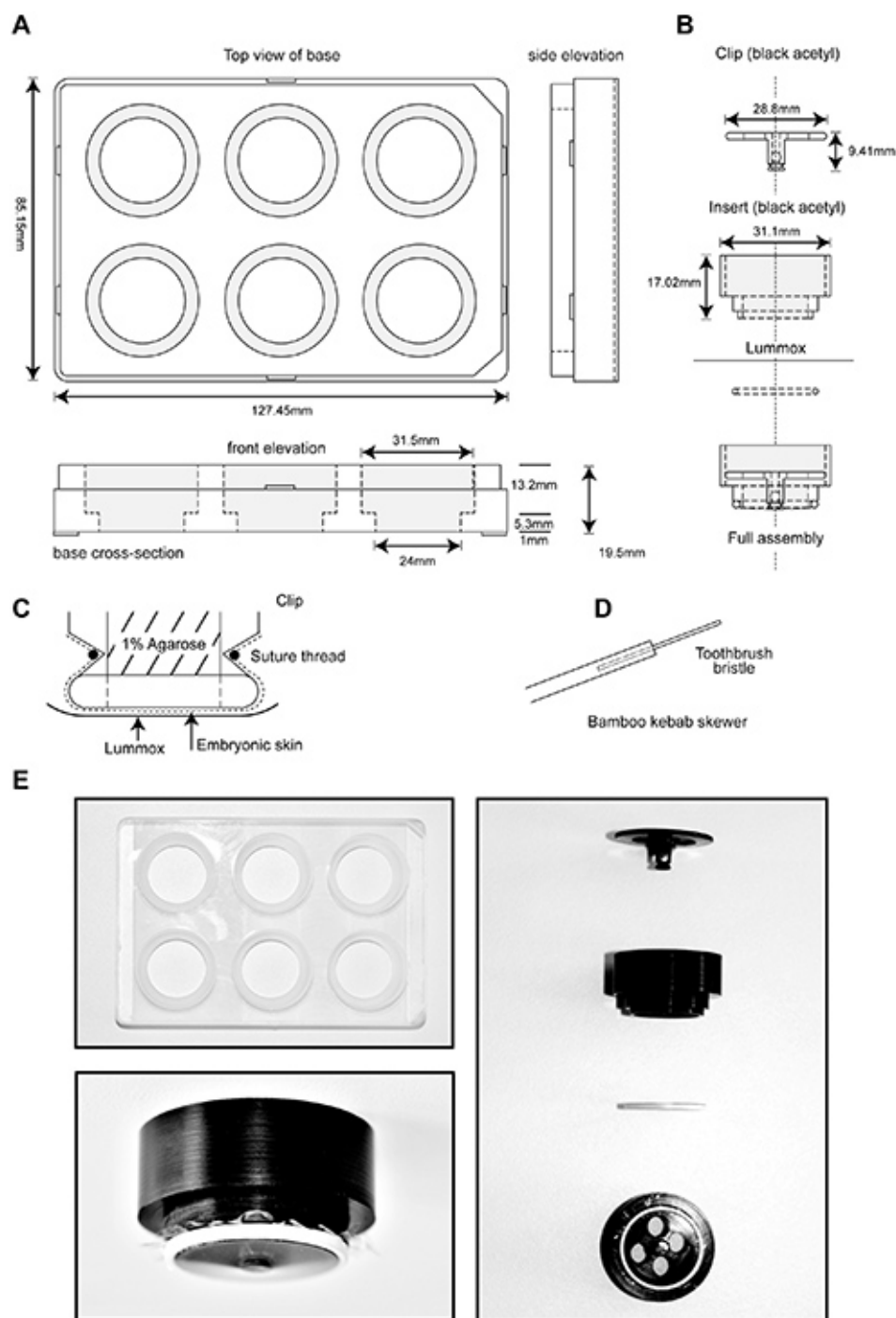


Figure 1. Equipment and setup. **A:** The live imaging chamber base consists of a clear perspex housing with identical outside dimensions to a 6-well culture dish. It contains six holes in which the 6 individual chamber assemblies can slot. **B:** Each insert consists of 4 components; an imaging clip, an imaging insert, a lummoX membrane, and an O-ring. The clip and the insert are lathed from black acetyl plastic, we found this to have the lowest autofluorescence of the plastics we tested. The O-ring is made from PVC and is used to clamp the lummoX membrane onto the bottom of the insert forming the air liquid interface (ALI). The imaging clip (black acetyl) has several holes to allow circulation of culture medium. **C:** The central shaft of the clip is filled with 1% agarose before use to provide a firm flat surface on which to mount the skin. The skin is attached to the clip with suture thread before being pushed into the insert, sandwiching the skin between the agarose and lummoX membrane. The insert is then filled with culture medium. **D:** To dissect and manipulate the embryonic skin a single tooth brush bristle is mounted into a slit in the flat end of a bamboo kebab skewer. A pair of hair sticks can be used in combination with fine forceps to dissect embryonic skin and mount in the imaging chamber. **E:** Photographs of the components detailed in A-C. [Please click here to view a larger version of this figure.](#)

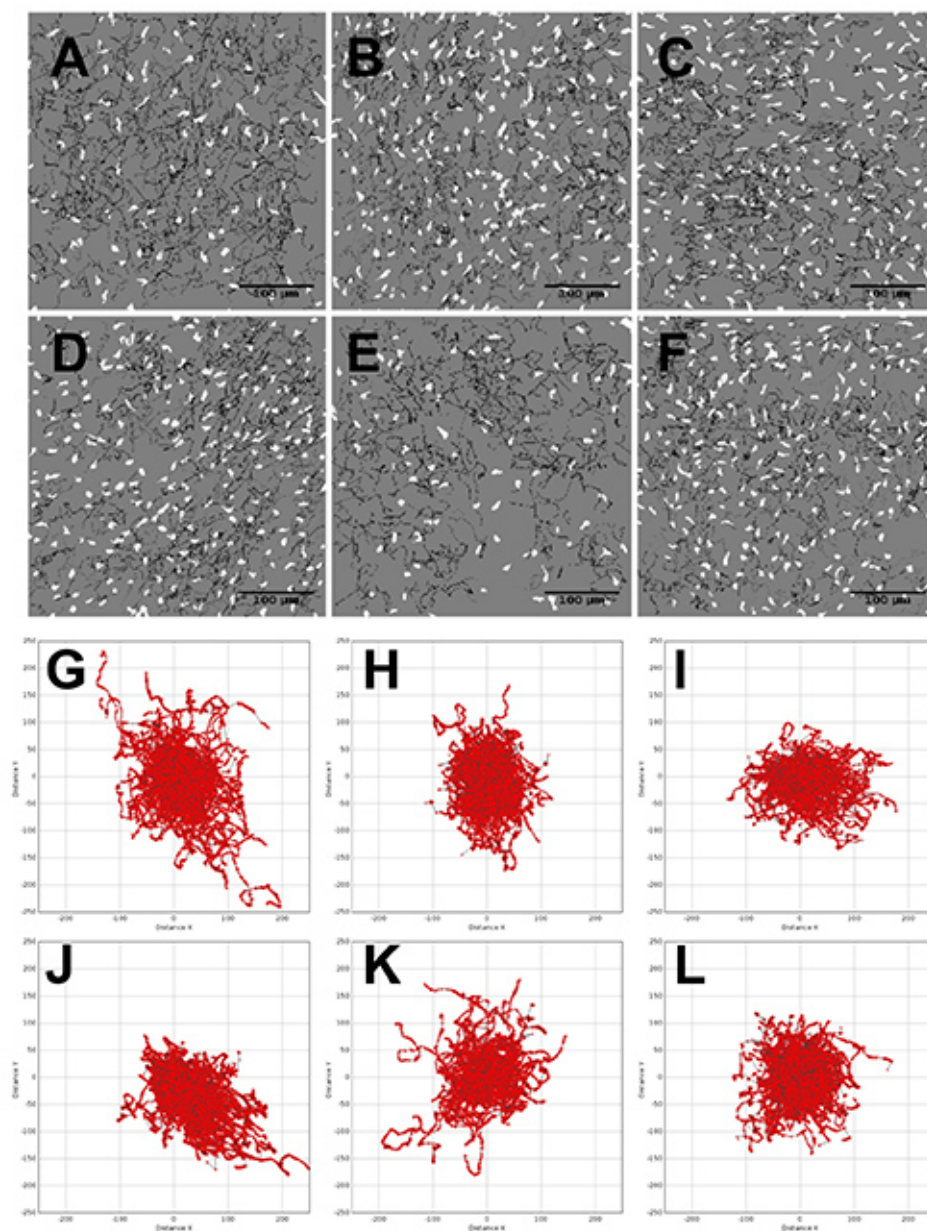


Figure 2. Representative results of 6 movies recorded in parallel. A-F: Tracking results from 6 movies recorded in parallel to demonstrate the capabilities of the system. Each movie was recorded at 2-min time points for a total of 18 hr (see **Movies 1-3** for the corresponding movie of panels A and G). The positions of the cells in frame one of the movie are shown and the tracking results are superimposed on top. **G-L:** Summary of the tracking results plotted as zeroed tracking data.

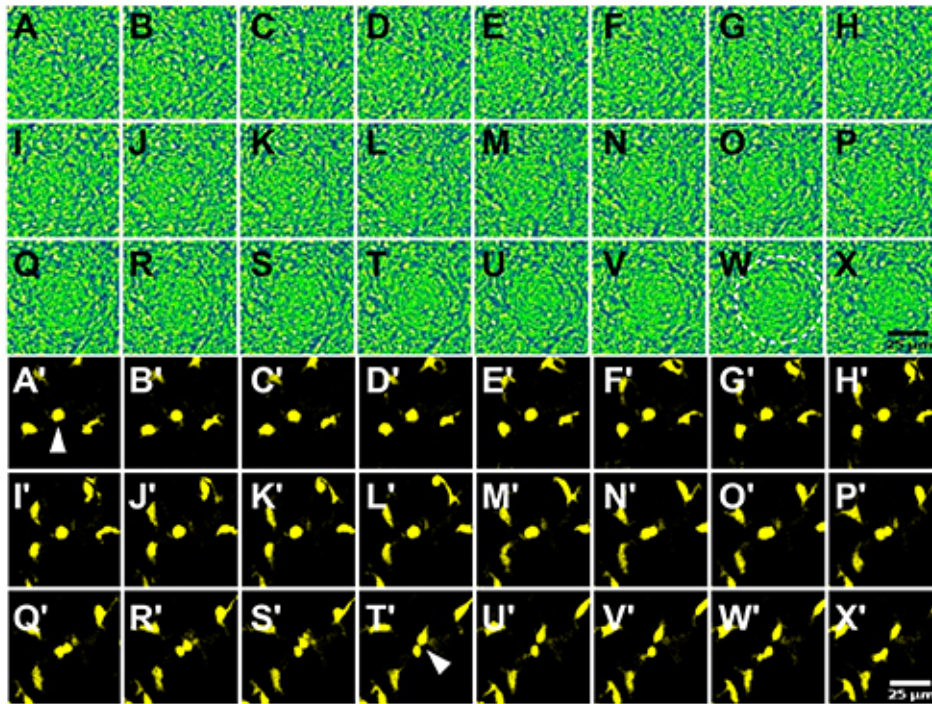


Figure 3. Representative images from time-lapse movies of hair follicle development and of a melanoblast undergoing mitosis. A-X: Selected, cropped frames from **Movie 1**. Primary hair follicles become visible over the period of skin culture from 0-18 hr. An LUT was applied to the images. **A'-X':** Selected, cropped frames from **Movie 2**. Representative images of melanoblasts migrating in the developing epidermis and an individual cell (see arrow in A') undergoing mitosis (arrow in T'). [Please click here to view a larger version of this figure.](#)

Movie 1: Representative result of the transmitted light channel from an 18-hr time-lapse experiment. The primary hair-follicle pattern, although not evident at the start of the movie, becomes more prominent as the culture progresses.

Movie 2: Representative result of the YFP channel from an 18-hr time-lapse experiment. The melanoblast population is a highly dynamic and migratory population. Rare stationary cells are usually undergoing mitosis (see **Figure 3**).

Movie 3: Representative result of the composite channel from an 18-hr time-lapse experiment. The melanoblast population can be seen to migrate within the developing epidermis and interact with the developing hair follicles.

Discussion

We describe a method to culture embryonic skin that is particularly amenable to live-cell imaging on inverted confocal microscopes. The method includes recent improvements that allow 6 cultures to be imaged in parallel and removes the reliance on matrigel and Nuclepore membranes of the original method⁶. The crucial technical difference from similar techniques is the use of a gas-permeable lumox membrane to establish an air liquid interface and also to act as a coverslip. We have successfully maintained the cultures with continuous confocal imaging for up to 48 hr. The high resolution and excellent signal to noise ratio of the confocal images allows automated cell tracking to analyze melanoblast behavior in the resulting time-lapse movies.

Using this technique it is possible to investigate the dynamics of melanoblast dispersal and study their interaction with the epidermis and the developing hair follicles. The critical steps in the protocol are to carry out a careful dissection that does not damage the developing epidermis and to mount the skin samples dermal side against the agarose without kinks or stretches. The procedure described in this paper works best between E13.5 and E15.5. Before E13.5 the skin is very fragile and after E15.5 it is relatively thick and hard to penetrate by confocal microscopy. The capabilities of the microscope system used are also very important. We typically use a Nikon A1R confocal microscope equipped with Nikon's proprietary perfect focus system (PFS). Using PFS greatly reduces the number of bad movies caused by stage drift, similar systems are available for other platforms.

One disadvantage of this technique is the requirement for specialized culture equipment. However the design is simple and the chamber could be easily assembled by any small technical workshop. In summary we present a simple method that should be of broad use for studies of embryonic skin development and melanoblast behavior. It is anticipated that the technique could also be modified for other scenarios where an air-liquid interface is a requirement for successful culture.

Disclosures

The authors have nothing to disclose.

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