Chapter 9

Fate-Patterning of 2D Gastruloids and Ectodermal Colonies Using Micropatterned Human Pluripotent Stem Cells

George Britton, Sapna Chhabra, Joseph Massey, and Aryeh Warmflash

Abstract

In the developing mammalian embryo, intercellular signaling allows cells to self-organize to create spatial patterns of different cell fates. This process is challenging to study because of the difficulty of observing or manipulating embryos on the spatial and temporal scales required. In vitro models can provide a complement to in vivo systems for addressing these issues. These models are also the only windows we have into early human development. Here we provide protocols for two systems based on differentiating human pluripotent stem cells in micropatterned colonies on defined size and shape. The first model replicates the patterning of the germ layers at gastrulation, while the second replicates the medial-lateral patterning of the ectoderm. These systems allow study of how signaling underlies self-organized patterning at stages of development which are otherwise inaccessible.

Key words Self-organization, Signaling, Human embryonic stem cells (hESCs), Micropatterns, Gastruloids, Ectoderm, Cellular communication, Tissue patterning

1 Introduction

Cell-to-cell communication via chemical and mechanical signals is integral to the formation of a spatially patterned organism. The interplay between signaling and tissue patterning is technically very challenging to study in a developing mammalian embryo. Micropatterning technology provides a platform to develop simplified, in vitro models of embryonic development that recapitulate tissue patterning. In these models, cells' attachment is restricted to regions of defined shapes and sizes [1]. Although submicron resolution can be achieved with micropatterning techniques, for tissue-patterning experiments, cells are typically confined to colonies in the range of 0.1–1 mm, the approximate size of a mammalian

George Britton, Sapna Chhabra, and Joseph Massey authors contributed equally to this work.

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embryo at gastrulation stage, or of an organ during its initial patterning. Spatial confinement has two advantages: First, controlling colony size removes variability from outcomes so that consistent patterns of signaling and cell fate are formed. Second, confinement leads to increased cell density, so that in micropatterns, but not in similarly sized colonies in standard culture, the density can be made to mimic that of the embryo. This in turn allows cell communication and self-organization similar to that which occurs in vivo. For instance, micropatterned 2D stem cell colonies, stimulated with appropriate growth factors or inhibitors, recapitulate gastrulation and ectodermal patterning in vitro [2, 3]. These simplified models provide a controlled, high-throughput and highly reproducible platform to quantitatively examine the role of signaling in patterning.

Previously, we have shown that micropatterned human embryonic stem cell (hESC) colonies, stimulated with BMP4 ligand, self-organize to form radial patterns of the three germ layers, thus recapitulating gastrulation in vitro (2D gastruloids) [2]. Starting from colony edge, the patterns comprise rings of trophectoderm, endoderm, and mesoderm with either ectoderm [2] or pluripotent cell-fates [4] at the colony center. This gastrulation protocol has been reproduced in other labs [5, 6], and a comparable system for mouse gastrulation has been developed [7]. The relative position of germ layers and the chemical signaling cascade underlying patterning are the same in micropattern gastrulation assays and the gastrulating mouse embryo [4, 8, 9]. Thus, these micropatterned systems provide a good platform to dissect the relationship between signaling and patterning, and can be utilized to uncover common and species-specific mechanisms underlying germ-layer patterning. In recent studies, we used the human gastrulation assay to show that dynamic waves of WNT and NODAL signaling, in the absence of an underlying spatial signaling gradient, control germ layer patterning [4, 10]. This suggests that the combinatorial dynamics of multiple signaling pathways, and not a concentration threshold in signaling, governs patterning during gastrulation.

Recently, we adopted micropatterning technology to create a system that recapitulates the medial-lateral (ML) patterning of the ectodermal germ layer [3]. This system provides an opportunity to understand how this layer is patterned into functionally distinct cell types, a topic of both fundamental interest and medical relevance as several of these fates are the subject of intense studies for regenerative therapies. Micropatterned hESCs are differentiated toward the ectodermal lineages, and patterning is then induced with BMP4. In response, the cells self-organize to form radial patterns containing the same cell fates in the same organization as found along the ML axis of mammalian embryos. Starting from colony edge, the patterns comprise surface ectoderm, placode, neural crest, and neural cell fates. This patterning is controlled by relative levels of BMP and WNT signaling. We used the information gained from the

micropatterned platform to improve differentiation protocols for the placodal cell fate. Thus, micropatterned assays can provide mechanistic insights into the signaling dynamics underlying patterning, with direct implications for improving differentiation protocols for regenerative medicine.

We have previously published a protocol for micropatterned 2D gastruloids in defined media [11]. Here, we modify that protocol for a 96-well format, include improvements for long-term storage of laminin-coated micropatterned surfaces, and introduce the protocol for ectoderm patterning.

2 Materials

2.1 Cell Culture

2.1.1 2D Gastruloid and Ectoderm Patterns

1. Human pluripotent stem cells. We routinely use ESI017 (ESI-BIO) and RUES2 (Ali Brivanlou, Rockefeller University) as well as induced pluripotent cells from the Coriell collection.
2. mTeSR1 culture media kit (basal media and 5× supplement) (STEMCELL Technologies; 85870).
3. Accutase (Fisher Scientific; NC9839010) for single-cell suspensions.
4. ROCK Inhibitor (RI) Y-27632 (Fisher Scientific; 50-175-998).
5. 35 mm Nunc Cell Culture/Petri Dish (Fisher Scientific; 1256591) for routine culture.
6. Cell culture incubator with controlled humidity and 5% CO_2.
7. Biological safety cabinet (Laminar Flow Hood).
8. 70% Ethanol for sterilizing work surfaces and tools.
9. 1, 5, 10, and 25 mL sterile serological pipettes and pipettor.
10. Micropipette tips with barrier and micropipettor.
11. PBS without calcium and magnesium (Caisson Labs; PBL01-6X500ML).
12. Dulbecco's Phosphate-Buffered Saline (DPBS), 1× with calcium and magnesium (VWR; 45000-430).
13. Inverted tissue culture microscope with phase contrast.
14. Hemocytometer.
15. Cell culture centrifuge.
16. Nalgene Rapid-Flow sterile disposable filter units with PES membrane (ThermoFisher; 569-0020).
17. Penicillin-streptomycin (Life Technologies; 15140-148).
18. Recombinant human BMP-4 (Fisher Scientific; 314BP050).
19. Fluoromount-G (Southern Biotech; 0100-01).
20. Microslides (VWR; 16004-382).

2.1.2 Additional Reagents Required for Ectoderm Pattern

1. Selective inhibitor of ALK4, Alk5, and ALK7. SB431542 (Stemgent; 04-0010-05).
2. DMEM-F12 (VWR; 45000-344).
3. Neurobasal media (Life Technologies; 21103-049).
4. N2 supplement 100× (Life Tehnologies; 17502048).
5. B27 supplement without vitamin A 50× (Life Technologies; 12587010).
6. Glutamax 100× (Life Technologies; 35050061).
7. ß-Mercaptoethanol (Fisher Scientific; 21985023).
8. WNT secretion inhibitor IWP2 (Stemcell Technologies; 72124).

2.2 Micropatterning

1. 96-Well micropatterned plate (CYTOO; 20-950-00) or chip (CYTOO; 10-021-00-18)
2. PBS with calcium and magnesium (VWR; 45000430).
3. Recombinant human laminin 521 (Biolamina, R021599/X0086842).
4. A multichannel micropipettor for high-throughput experiments.

2.3 Immunofluorescence and Imaging

1. 4% Paraformaldehyde (wt/vol). Prepared from 16% stock (EM Sciences; 15710) diluted in PBS.
2. Blocking solution: 3% normal donkey serum (EMD Millipore; S30-100ML) with 0.1% Triton X-100 (Sigma; 1001843780) in PBS. Stores for 1 week at 4 °C.
3. PBST washing solution: 0.1% Tween-20 (Sigma; P1379) in PBS.
4. Human Brachyury antibody, goat-derived (R&D Systems; AF2085), 1:300 dilution.
5. Human Sox2 antibody, rabbit-derived (Fisher Scientific; 5024S), 1:200 dilution.
6. Human CDX2 antibody, mouse-derived (Abcam; AB15258), 1:50 dilution.
7. Human N-CAD antibody, mouse-derived (Sigma; C2542), 1:100 dilution.
8. Human E-CAD antibody, rabbit-derived (Fisher Scientific; 31955), 1:200 dilution.
9. Human SOX9 antibody, goat-derived (R&D Systems; AF3075), 1:200 dilution.
10. 4,6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (Life Technologies; D1306).

11. Secondary antibodies: Donkey anti-Mouse Alexa Fluor 488, anti-Goat Alexa Fluor 555, and anti-Rabbit Alexa Fluor 647 (Thermo Fisher; A-21202, A-21432, and A-31573), dilution 1:500.
12. Inverted fluorescence microscope for imaging (e.g., Olympus FV1200).

3 Methods

Embryonic gene expression patterns for both gastruloids and ectoderm have successfully been generated on CYTOO micropatterned coverslips and 96-well plates. There are advantages and disadvantages to each micropattern format, and we suggest potential users to select the one that best suits their research question. For example, the 96-well plate is easily amendable to live-cell imaging, and well suited for multiplexing experimental conditions, technical repeats, and primary antibody cocktail combinations. Additionally, the benefit of lower media volumes to conduct a single experiment translates to using fewer resources such as recombinant laminin for coating, antibodies for immunohistochemistry and small molecules and ligands for differentiation protocols. One potential drawback to the 96-well plate is the limited number of shapes, sizes, and replicate number of micropatterned surfaces in each well. On the other hand, the larger culture surface on CYTOO chips allows one to study, in a single experiment, the role of size and shape during embryonic patterning events. However, the large media volume needed to conduct a single experiment on CYTOO chips means it is far less economical and difficult to scale for experiments that require multiple experimental conditions and controls. In the method that follows below, we provide detailed procedures to coat, wash, and seed hESCs to each micropattern format. We then provided the necessary signaling conditions and media volumes to generate either gastruloid or ectodermal gene expression patterns on CYTOO chips and 96-well plates.

An important consideration for any experiment conducted on micropatterned surfaces (CYTOO chip or 96-well plate) is that the position and intensity of cell fate markers in resulting patterns is sensitive to experimental variation. These include minor variations in initial seeding density, the duration of differentiation, and the duration of each step of immunofluorescence staining. For these reasons, we recommend always making comparisons against controls that are done alongside each experimental condition, and not at separate times.

3.1 Base Media Preparation

1. mTeSR
   (a) Thaw mTeSR 5× supplement overnight at 2–8 °C. Mix thoroughly once completely thawed.
   (b) Working in a sterile culture hood, add 400 mL of mTeSR basal medium and 100 mL of mTeSR 5× supplement to 500 mL Nalgene Rapid-Flow sterile filter unit.
   (c) Connect and apply vacuum to Rapid-Flow unit for sterile filtration of mTeSR.
   (d) Store filtered mTeSR at 4 °C for up to 2 weeks or at −20 °C for up to 6 months.
2. N2B27
   (a) Thaw N2 and B27 supplements at room temperature.
   (b) Working in a sterile culture hood add 250 mL of DMEM/F12, 250 mL of neurobasal media, 5 mL glutamax 100×, 5 mL B27 50× supplement, 2.5 mL N2 100× supplement, and 0.5 mL ß-mercaptoethanol to 500 mL Nalgene Rapid-Flow Sterile filter unit.
   (c) Connect and apply vacuum to Rapid-Flow unit for sterile filtration of N2B27 media.
   (d) Store-filtered N2B27 media at 4 °C for up to 4 weeks or at −20 °C for up to 6 months.

3.2 Coat Micropatterned Surface with Laminin and Seed Cells

All of these procedures should be done inside the tissue culture hood while observing proper aseptic technique unless otherwise noted. Media and PBS++ should be at room temperature or pre-warmed to 37 °C.

For experiments conducted in a 96-well plate, it is important to consider that unused wells can be used in subsequent experiments. We have found hESC attachment and their potential to form gene expression patterns on previously unused micropatterned surfaces remain unaffected. To increase the lifespan of the 96-well plate, we store plates at 4 °C between experiments and only prepare wells for seeding as needed. A few of our plates have gone through upwards of five cycles of experiments spread over more than 6 months. To be clear, we never reuse the same wells. It is the previously unused wells which remain available for subsequent experiments.

3.2.1 Prepare Laminin Coated Micropattern Surface

96-Well CYTOO Micropattern Plate

1. Add 5 μg/mL laminin 521 (diluted in PBS with Calcium and Magnesium, hereafter PBS++) to each well. For a 96-well plate, we typically use 100 μL per well (*see* **Note 1**).
2. Incubate plate at 37 °C for 2.5 h.
3. Gently wash each well with PBS++ with the pipette tip placed against the edge of the well. A multichannel pipettor can be used for multiple wells.

(a) Begin with four partial washes by adding and removing 250 μL PBS++. A remaining 100 μL PBS++ should be present between washes.

(b) Remove the remaining 100 μL PBS++ and perform one complete wash by adding and withdrawing 250 μL PBS++. Perform this step quickly to be sure micropattern surfaces do not dry between washes.

(c) Add 100 μL PBS++ to each coated and washed microwell. Micropatterns are available to use immediately or can be stored in PBS++ at 4 °C for up to 2 weeks.

CYTOO Micropattern Chip

1. Place each CYTOO micropattern chip face up in one 35 mm plate.
2. Add 5 μg/mL laminin 521 diluted in PBS++ to each 35 mm plate. Each chip will require 2 mL of diluted laminin solution.
3. Incubate the chip at 37 °C for 2.5 h.
   (a) Press the corners of the chip to the surface of the 35 mm plate with a 1-mL pipette tip in case the micropattern chip begins to float upon addition of laminin-PBS++ solution.
4. Gently wash each chip with PBS++.
   (a) Begin with five partial washes by adding and removing 6 mL of PBS++ using a 10-mL serological pipette. A remaining 2 mL PBS++ should be present between washes.
   (b) Remove the remaining 2 mL PBS++ and perform one complete wash by adding and withdrawing 8 mL of PBS++. Perform this step quickly to be sure micropattern surfaces do not dry between washes.
   (c) Add 2 mL PBS++ to each coated and washed micropatterned chip. The chip is available to use immediately or can be stored in PBS++ at 4 °C for up to 2 weeks.

3.2.2 Seed Cells

1. Prepare single cell suspension of hESCs for seeding.
   (a) Calculate the total volume of mTeSR media with 10 μM ROCK inhibitor Y-27632 (RI) needed to harvest, count, and seed cells to micropattern surfaces. Volume of media required for each step: ***Harvesting cells***: Each plate will require 1 mL of mTeSR with RI to harvest cells following accutase treatment (e.g., need 2 mL of mTeSR with RI to harvest cells from 2.35 mm plates). ***Counting cells***: To resuspend cells following centrifugation, one can expect to use 0.5–1 mL of mTeSR with RI for every ~1×10^6 cells. ***Seeding cells***: The media volume will scale with the number of wells coated in a 96-well plate (100 μL mTeSR+RI/well) and/or the number of coated chips (2 mL mTeSR+RI/chip).

(b) To harvest cells, aspirate media from plate with adherent hESCs and wash with PBS without calcium and magnesium twice.

(c) Add 500 mL of accutase to each 35 mm plate and incubate at 37 °C for 5 min or until cells have detached.

(d) Add 1 mL of mTeSR with RI to each 35 mm plate to dilute accutase. Use pipette to break hESC colonies into a single cell suspension.

(e) Transfer cell suspension to 15 mL tube and pellet cells in centrifuge at 180 RCF for 4 min.

(f) Aspirate media and resuspend cells in 0.5–1 mL mTeSR with RI (10 μM) for every ~1×10^6 cells collected (Estimated. For reference, a nearly confluent 35 mm dish typically contains about 3×10^6 hESCs.)

(g) Count the number of cells/mL using a hemocytometer.

2. Aspirate/remove PBS++ from wells in CYTOO plate/chips.
3. Seed cells to micropatterned surfaces. The number of cells will depend on the micropatterned format (96-well plate vs. chip) and pattern model (gastruloid vs. ectoderm). ***For 96-well plate***, seed 180,000 cells/well for gastruloid patterns or 120,000 cells/well for ectodermal patterns. For both models, cells are seeded to wells using 100 μL of mTeSR plus RI. ***For chips***: seed 1.8×10^6 cells/chip for gastruloid patterns or 1.2×10^6 cells/chips for ectodermal patterns. For both models, cells are seeded to chips in 35 mm plates using 2 mL of mTeSR plus RI.
4. Place chips or 96-well plates in the incubator for 45 min at 37 °C. Note: incubation time can go up to 1.5 h if needed (*see* **Note 2**).
5. Prepare differentiation media prior to washing cells.

3.3 Wash Nonspecifically Bound Cells

1. Completely wash cells with PBS (250 μL/well of a 96-well plate and 1 mL/chip in 35 mm dish) without calcium and magnesium (PBS– –) 1–6 times until the majority of cells outside of the pattern have been removed. First washes should be gentler and against the side of the well of a 96-well plate or 35 mm dish. It is best practice to continually check progress on phase contrast microscope and gradually pipette more aggressively as needed. It is not necessary to remove all of the non-patterned cells at this point; many will die and detach once RI is removed at a later step (Fig. 1).

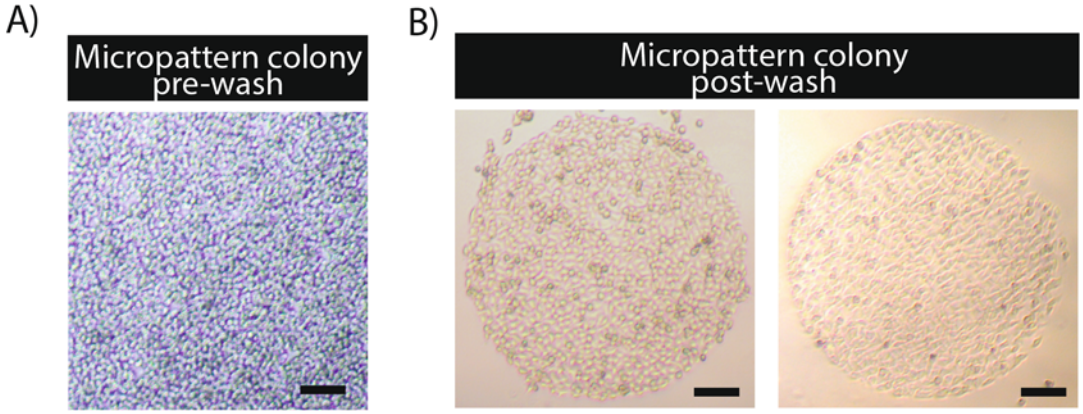

Fig. 1 Images of hESCs on micropatterns at the time of seeding. (**a**, **b**) Representative phase contrast images of hESCs seeded to a micropattern surface before (**a**) and after (**b**) washing. Colony diameter: 700 μm. Scale bar: 100 μm

3.4 Differentiate

3.4.1 Gastruloid Differentiation

Proceed to either gastruloid differentiation or ectodermal differentiation

The induction media is introduced only at the beginning and uses a volume of 200 μL for 96-well plates and 2 mL for chips.

1. After washing micropatterned cell colonies, replace PBS with mTeSR containing 50 ng/mL BMP4 and Pen/Strep (1%). Note that this media no longer contains RI. For 96-well plates, use 200 μL media. For chips use 2 mL. media.
2. Incubate at 37 °C for 42–48 h.

3.4.2 Patterned Ectoderm Differentiation

There are several treatment protocols that provide different ectoderm patterns described in our initial report [3]. Here we describe one of the protocols that results in patterns consisting of placodes, neural crest, and neural cell fates.

Media is changed daily with the media used on each day described below. Use 100 μL/well of a 96-well plate and 2 mL/chip in a 35 mm plate. Note RI is absent throughout the induction protocol.

1. After washing micropatterned cell colonies, replace PBS with mTeSR supplemented with SB431542 (10 μM) and pen/strep (1%).
2. After 24 h, exchange the media for N2B27 supplemented with SB431542 (10 μM) and pen/strep (1%) for the following two nights.
3. After three nights of induction with SB431542, introduce 1–3 ng/mL of BMP4 to N2B27 media with 10 μM SB431542 and pen/strep (1%).

4. On the following day, BMP4 signaling is maintained while WNT secretion is inhibited with N2B27 media containing 1–3 ng/mL BMP4, 10 μM SB431542, 3 μM IWP2, and pen/strep (1%).
   (a) Use the same concentration of BMP4 as the prior day.
5. Cells are fixed on the sixth day.

3.5 Fix Cells, Stain for Immunofluorescence, and Image

PFA should only be used in a chemical safety hood. PFA fixation and immunofluorescence staining can be performed using standard techniques for cell culture while being especially careful while pipetting not to disturb patterned cells.

3.5.1 Common to CYTOO 96-Well Plates and Chips

1. Fix cells by replacing media with 4% Paraformaldehyde (Wt/Vol; 100 μL/well of 96-well plate or 1 mL/chip in 35 mm dish) in PBS and incubating at room temperature for 20 min.
2. Aspirate PFA and wash cells with PBS twice (300 μL/well of 96-well plate and 2 mL/chip in 35 mm dish).
3. Add blocking solution (100 μL/well of 96-well plate and 1 mL/chip in 35 mm plate) to cells and incubate at room temperature for 1 h.
4. Replace blocking solution with primary antibodies diluted in blocking solution (50 μL/well of 96-well plate and 1 mL/chip in 35 mm dish) and incubate at room temperature for 1 h or overnight at 4 °C.
5. Perform three 20-min washes at room temperature with PBST (300 μL/well of 96-well plate and 2 mL/chip in 35 mm dish).
6. Replace final PBST wash with secondary antibody solution diluted in blocking solution (50 μL/well of 96-well plate and 1 mL/chip in 35 mm dish) and incubate at room temperature for 30 min.
7. Wash three more times with PBST (same volume as **step 5**) for 20 min each at room temperature.
8. Replace PBST wash with DAPI diluted in PBS to a final concentration of 300 nM (50 μL/well of 96-well plate and 1 mL/chip in 35 mm dish) and incubate at room temperature for 30 min.
9. Perform two 5-min washes with PBS (300 μL/well of 96-well plate and 2 mL/chip in 35 mm dish).
10. Replace final PBS wash with PBS (200 μL/well of 96-well plate and 2 mL/chip in 35 mm dish).
11. Mount CYTOO chips on a slide.

(a) Add 30 μL of fluoromount to a clean microslide and carefully transfer the CYTOO chip to the slide with its face oriented down on the microslide.

(b) Let each sample dry in a dark environment overnight.

12. Image cells using a fluorescent microscope (Fig. 2).

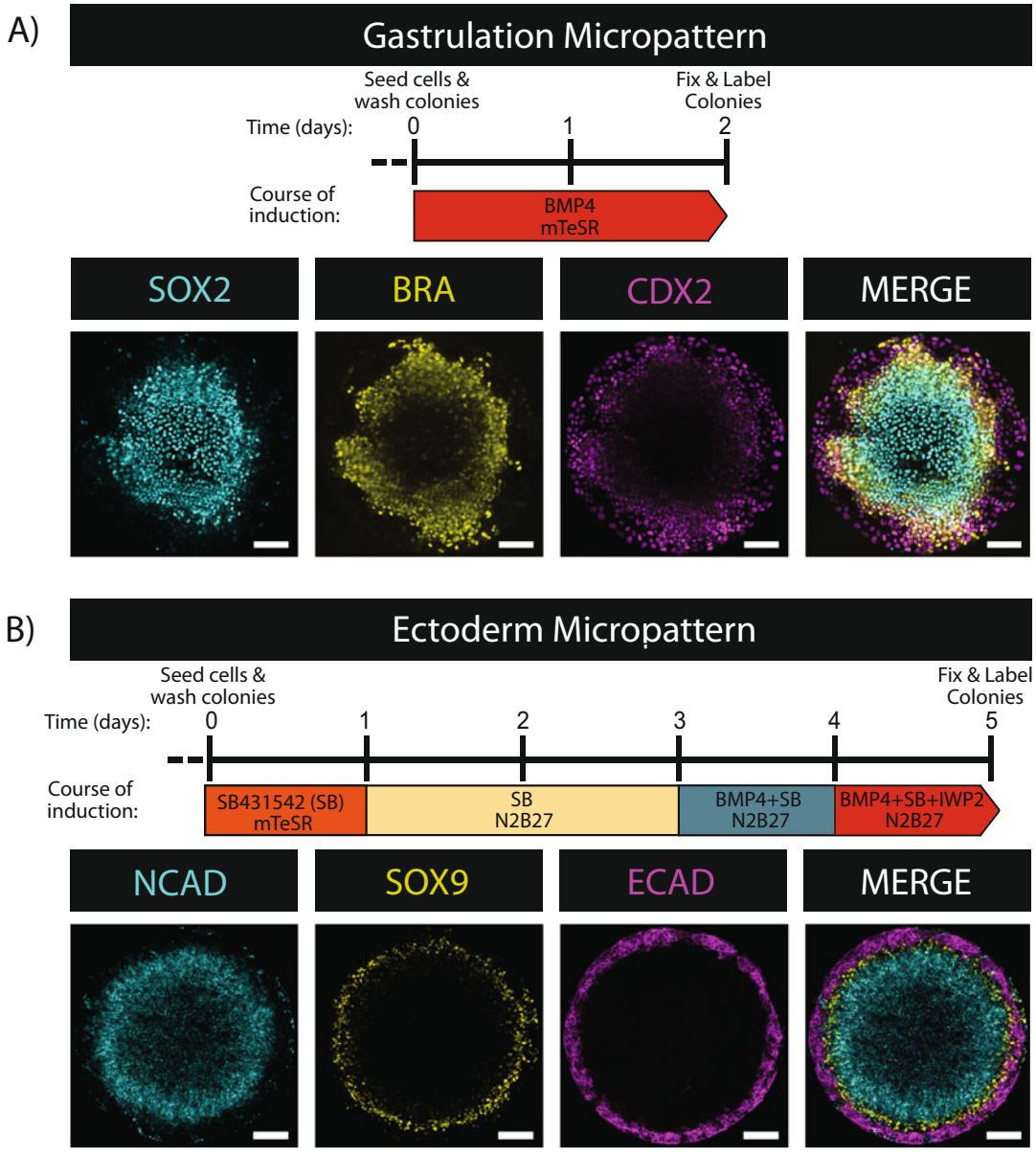

Fig. 2 Induction protocol and fate patterning in 2D gastruloids and ectodermal patterns. (**a**, **b**) Representative images of colonies with the indicated single or merged immunolabels following a gastruloid (**a**) or ectoderm (**b**) induction protocol with 50 ng/mL of BMP4 (**a**) or 1 ng/mL of BMP4 (**b**). The base media for each day is indicated below the cocktail of supplied agonists and inhibitors. Colony diameter: 700 μm. Scale bar: 100 μm

4 Notes

1. Sometimes batch-to-batch variability in laminin 521 results in the need for empirically determining the optimal coating concentration, though this is rare. We typically find the optimal concentration is between 5 and 20 μg/mL.
2. Number of seeded cells may need to be determined empirically for each cell line. A lower number of cells is seeded for ectoderm patterning experiments due to the longer growth time, while for gastruloids the seeding density can be higher. For gastruloids in particular, it is important that patterns are completely full after seeding, and it is generally better to err on the side of having more cells than having too few.

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