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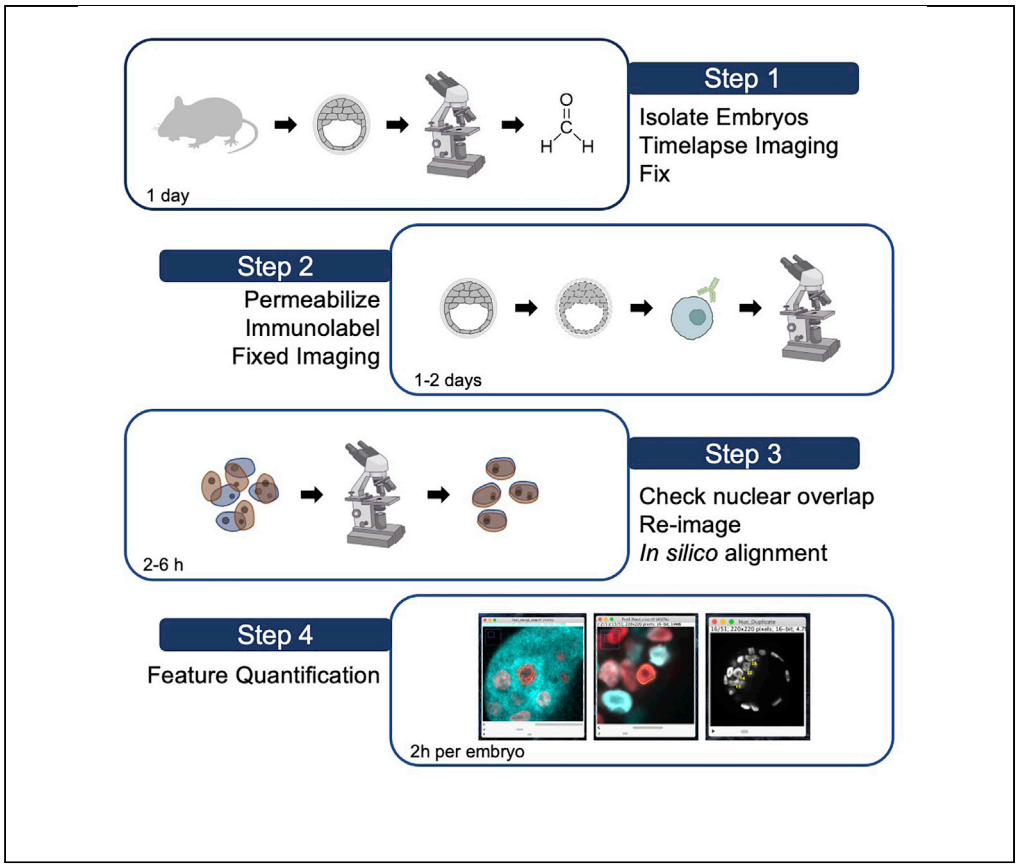
3D time-lapse microscopy paired with endpoint lineage analysis in mouse blastocysts



Determining how signaling dynamics relate to gene expression and cell fate is essential to understanding multicellular development. We present a unified live imaging and lineage analysis method that allows integrated analysis of both techniques in the same mouse embryos. This protocol describes the embryo isolation, confocal imaging, immunofluorescence, and *in silico* alignment required to connect time-lapse and endpoint measurements. By utilizing different biosensors and fixed readouts, this method allows interrogation of signaling dynamics that specify cell fates in developing embryos.

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Highlights

Protocol for time-lapse imaging followed by immunofluorescent staining in blastocysts

Iterative reimaging method facilitates *in silico* alignment of data

Image analysis examples guide ERK-KTR quantification strategies in live mouse embryos

Pokrass & Regot, STAR Protocols *2*, 100446
June 18, 2021
https://doi.org/10.1016/j.xpro.2021.100446

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3D time-lapse microscopy paired with endpoint lineage analysis in mouse blastocysts

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https://doi.org/10.1016/j.xpro.2021.100446

SUMMARY

Determining how signaling dynamics relate to gene expression and cell fate is essential to understanding multicellular development. We present a unified live imaging and lineage analysis method that allows integrated analysis of both techniques in the same mouse embryos. This protocol describes the embryo isolation, confocal imaging, immunofluorescence, and *in silico* alignment required to connect time-lapse and endpoint measurements. By utilizing different biosensors and fixed readouts, this method allows interrogation of signaling dynamics that specify cell fates in developing embryos.

For complete details on the use and execution of this protocol, please refer to Pokrass et al. (2020).

BEFORE YOU BEGIN

Set mouse breeding pair

Timing: 5 min (+ 4 days for embryo development)

This protocol describes a method for embryo derivation in which adult male and female mice are set in the same cage to allow natural mating.

Alternatives: Mouse embryos can be obtained for imaging by natural mating, mating with superovulated females (Liang et al., 2013), or *in vitro* fertilization (IVF) techniques (Chaigne, 2018). While superovulation and IVF can increase the number of embryos obtained, embryo viability tends to decline and other aspects of preimplantation development may be perturbed in these conditions (Van Der Auwera and D'hooghe, 2001).

Note: If transgenic embryos will be used in experiments, design a comprehensive plan of the crosses required to generate embryos of each genotype needed in the study before beginning experimentation.

Note: This method is expected to work with multiple biosensors, but it requires a nuclear marker in both the live and fixed imaging sections.

1. Select adult male and female animals of appropriate background and genetics for the experiment.

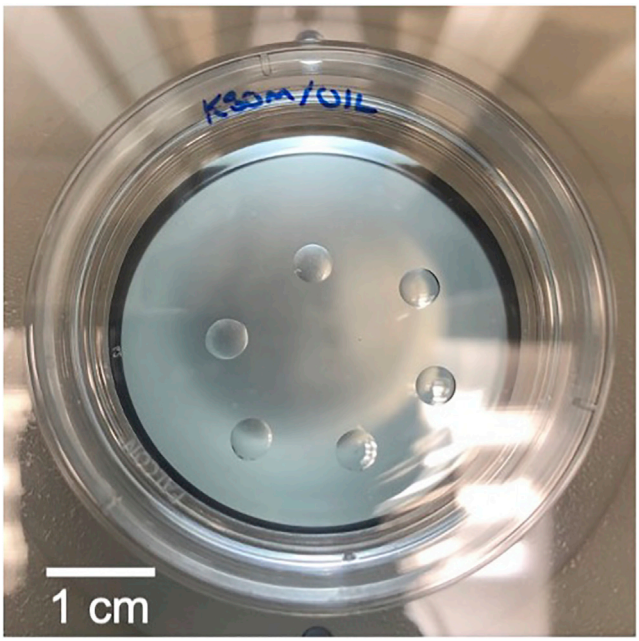

Figure 1. Image of KSOM drop arrangement on 60-mm dish for the embryo culture plate

Note: We recommend using females between 6 weeks and 6 months of age and males at least 8 weeks old.

2. Place male and female animals into a clean cage with food and water.
3. On the following morning before 10 AM, check females for mucosal plugs as evidence of coitus.

Note: Matings can be set up as one-to-one pairings or one male with multiple females. Multiple males should not be put in the same breeding cage as this can lead to aggressive behavior and fighting between animals.

Prepare embryo culture dishes

Timing: 30 min

4. Thaw 1 mL of KSOM at 37°C.

Alternatives: Powder KSOM formulations can be stored at 4°C before reconstitution and use the day of the experiment.

5. Pipette 5–6 drops of 10 μL KSOM onto a 60-mm tissue culture dish. The drops should be arranged on the plate such that they do not contact one another or the edge of the dish (Figure 1).
6. Completely cover the drops of KSOM with embryo-qualified mineral oil to prevent evaporation.
7. Carefully place plate in 5% CO_2 incubator and allow media to equilibrate for at least 30 min.

Note: Gases such as CO_2 diffuse slowly through mineral oil and thus longer incubations are required to allow equilibration before embryos are cultured in the medium (Kubie, 1927).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-NANOG	Cell Signaling Technology	Cat# 8822; RRID:AB_11217637
Goat polyclonal anti-GATA6	R&D Systems	Cat# AF1700; RRID:AB_2108901
Mouse monoclonal anti-CDX2	BioGenex	Cat# MU392; RRID:AB_2335627
Donkey anti-rabbit IgG (Alexa Fluor 405)	Abcam	Cat# ab175649; RRID:AB_2715515
Donkey anti-mouse IgG (Alexa Fluor 488)	Molecular Probes	Cat# A21202; RRID:AB_141607
Donkey anti-Goat IgG (Alexa Fluor 647)	Molecular Probes	Cat# A21447; RRID:AB_141844
Chemicals, peptides, and recombinant proteins		
EmbryoMax Advanced KSOM Embryo Medium	MilliporeSigma	Cat# MR-101-D
Mineral oil, suitable for mouse embryo cell culture	Sigma Aldrich	Cat# M8410
Formaldehyde	Fisher Scientific	Cat# PI28908
Phosphate buffered saline (PBS)	Quality Biological	Cat# 114-058-101CS
Triton X-100	Sigma Aldrich	Cat# X100-100ML
Glycine	Bio-Rad	Cat# 161-0718
Horse serum	Sigma Aldrich	Cat# H1138-500ML
Agar	Fisher Scientific	Cat# BP1423-500
M2 medium	Sigma Aldrich	Cat# M7167-50ML
Bovine serum albumin	Sigma Aldrich	Cat# A9647-100G
Experimental models: organisms/strains		
Mouse: ERK-KTR-LSL	Pokrass et al. 2020	JAX#035566
Mouse: ERK-KTR-LoxP	Pokrass et al. 2020	N/A
Software and algorithms		
ImageJ/Fiji	NIH	https://imagej.nih.gov/ij/index.html
Deposited data		
Integrated time-lapse and end-point datasets of ERK KTR Embryos	Pokrass et al. 2020	BioImage Archive, Accession S-BIAD28
Other		
Aspirator tube assemblies for calibrated microcapillary pipettes	Sigma Aldrich	A5177-5EA
Disposable borosilicate glass pasteur pipets	Fisher Scientific	Cat# 13-678-20C
New Brunswick Galaxy 170 R High Capacity CO_2 incubator	Eppendorf	Cat# 17334002
Nikon SMZ745 stereoscopic microscope	Nikon	Model: SMZ745
Tokai Hit ThermoPlate	Tokai Hit	Model: TPi-SMZSSX
Nikon Eclipse Ti Microscope	Nikon	N/A
Yokogawa CSU-W1 confocal scanner unit	Yokogawa	N/A
Photometrics Prime 95B sCMOS camera	Photometrics	N/A
Okolab humidified environmental control chamber	Okolab	Model: H301-K-FRAME

MATERIALS AND EQUIPMENT

- Mouth Pipette for Embryo Manipulation (Figure 2)

  Note: There are many ways to construct a mouth pipette like the one in Figure 2. A mouth piece and plastic tubing can be purchased from Sigma-Aldrich and modified to fit a variety of glass Pasteur pipettes. We pull Pasteur pipettes in-house according to previously described techniques (Behringer et al., 2014; Czechanski et al., 2014).

- Mammalian tissue culture incubator set at 37°C and 5% CO_2

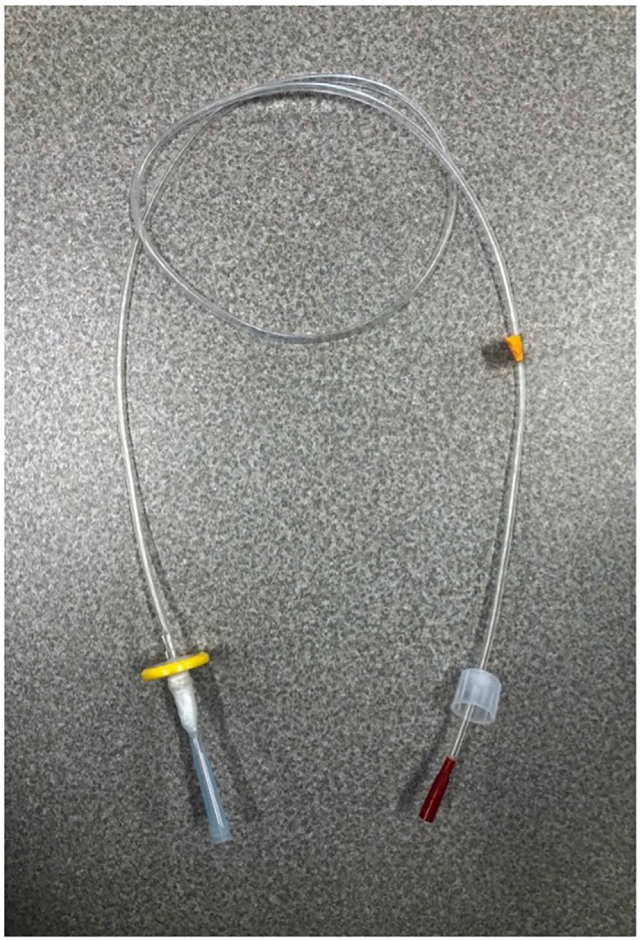

Figure 2. Image of assembled mouth pipet for embryo manipulation

- Nikon SMZ745 stereoscopic microscope

  Optional: A heated-stage (such as the Tokai Hit ThermoPlate model TPi-SMZSSX) can be fit to the stereoscopic microscope to help maintain stable temperatures during the embryo isolation protocol.

- Metamorph-controlled Nikon Eclipse Ti microscope with a Yokogawa CSU-W1 spinning disk confocal unit, Photometric Prime 95B sCMOS camera, and at least a 40× objective
- Okolab humidified environmental control chamber for microscope stage

PBX

Reagent	Concentration	Amount
PBS	n/a	9,990 µL
Triton X-100	0.1% v/v	10 µL
Total	n/a	10 mL

Store for up to one year at 20°C–25°C.

Permeabilization Buffer		
Reagent	Concentration	Amount
PBS	n/a	9,950 µL
Glycine	100 mM	75 mg
Triton X-100	0.5% v/v	50 µL
Total	n/a	10 mL
Store for one month at 4°C.		

Blocking Buffer		
Reagent	Concentration	Amount
PBS	n/a	9,800 µL
Horse Serum	2% v/v	200 µL
Total	n/a	10 mL
Store for one week at 4°C.		

Plate Coating Solution		
Reagent	Concentration	Amount
PBS	n/a	10 mL
Agar	1% m/v	100 mg
NaCl	0.9% m/v	90 mg
Total	n/a	10 mL
Microwave 30–60 s or until solids are dissolved. Store for one year at 4°C. Frozen solution can be microwaved to thaw.		

STEP-BY-STEP METHOD DETAILS

Embryo isolation

Timing: 1 h

Pregnant mice are euthanized and the uterus is dissected. Embryos are flushed from the uterine horns and collected for the experiment.

1. Euthanize pregnant females at E3.5 according to approved institutional animal care and use committee protocol.

   Note: Embryo staging can be done by checking plugs or by counting the number of cells. If checking plugs, E0.5 represents the day the plug was observed and if counting the number of cells, embryos of 32–64 total cells represent the E3.25-E3.5 blastocyst stage (Saiz et al., 2016).

2. Place mouse onto absorbent pad in dissection area in a supine position (abdomen facing up). Spray the animal with 70% ethanol (Figure 3A)
3. Using scissors, make a transverse incision in the skin and peritoneum crossing the midline of the animal.
4. Using forceps, move skin, fatty tissue, and intestines to reveal the uterus (Figure 3B).
5. Dissect the uterus by cutting just below the cervix and between the oviduct and ovaries. Transfer tissue to a 60-mm dish with PBS.

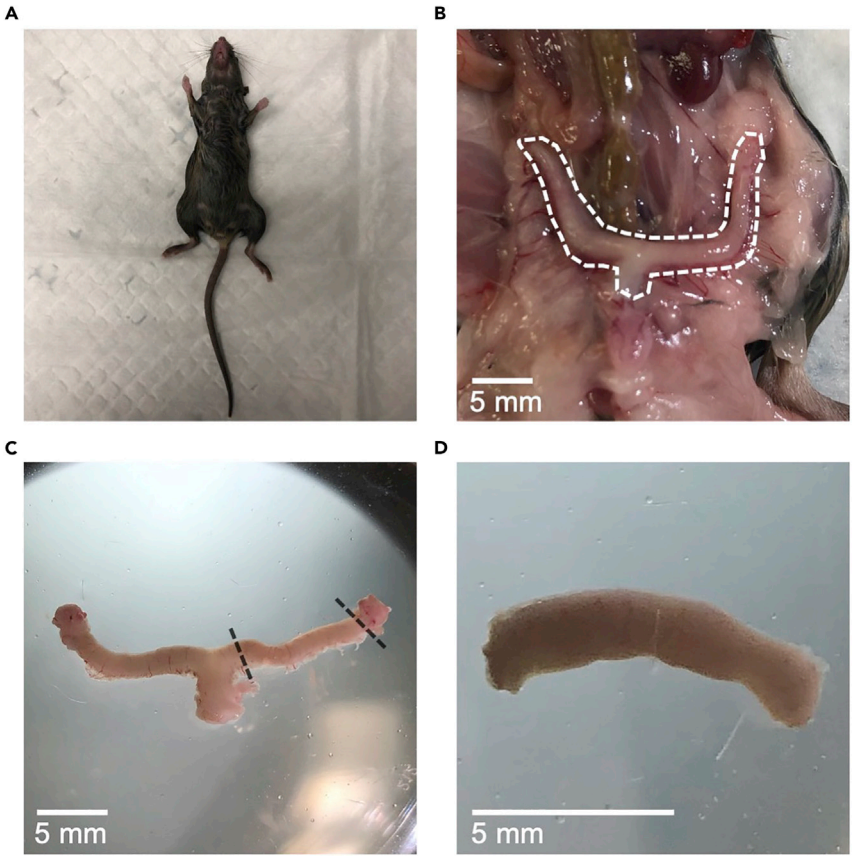

Figure 3. Uterine horn dissection for blastocyst collection

(A) Mouse placed in supine position.

(B) Visible uterus after adipose tissue and viscera have been removed. Uterus is outlined in dashed white line.

(C) Dissected uterus in PBS. Dashed lines indicate approximate region where cuts should be made.

(D) Uterine horn after it has been dissected from the oviduct and cervix.

Optional: Fatty tissue attached to the uterus can be removed at this stage with scissors. This can make subsequent steps of the dissection easier and it will be more difficult to trim away the fat once the uterine horns have been removed from the animal.

6. Dissect each uterine horn by cutting at the uterotubal junction (UTJ) and just before the horn meets the cervix (Figures 3C and 3D).
7. Using a transfer pipette, add ~300 μL warm M2 to a 35-mm dish. Place one uterine horn from the PBS to the drop of M2.
8. Fill a 1 mL syringe with warm M2. Insert the needle tip into the uterine horn near where the UTJ cut was made. Gently flush the uterine horn with 0.5 mL M2. You should observe some swelling of the tissue as it is filled with media.
9. Remove and dispose of the tissue that was just flushed with media and repeat the flushing step with the second uterine horn.
10. Allow approximately 1 min for embryos to settle to the bottom of the dish. Using a dissection microscope, scan the plate for embryos. [Troubleshooting 1]

Note: If dissecting multiple animals on the same day, this time can be used to flush the remaining uterine horns into fresh M2 medium.

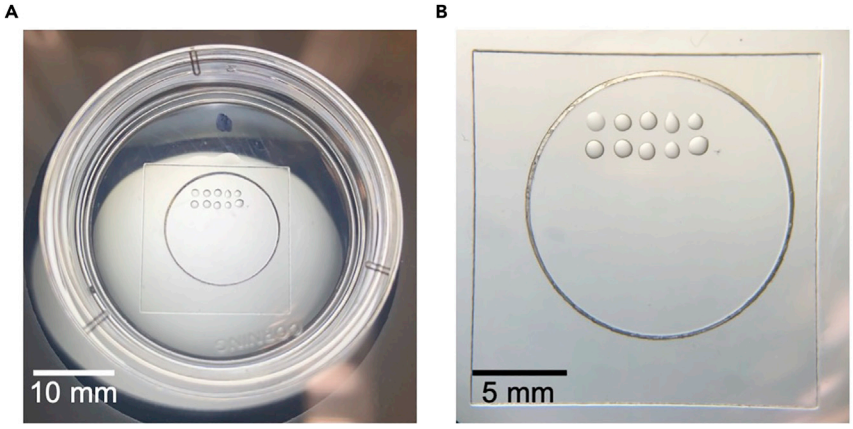

Figure 4. Embryo imaging plate

(A) KSOM drops arranged in grid on coverslip area and covered in mineral oil.

(B) Magnified image of coverslip area in (A) through view of stereomicroscope.

11. Transfer embryos from the M2 media into one drop of KSOM in the embryo culture dish.
12. Wash embryos by sequentially passing them into each of the KSOM drops.

Optional: Remove zona pellucida (ZP) by acid wash (described in detail in Nagy, 2006). ZP removal is particularly important for staining with some primary antibodies. Embryos denuded of ZP tend to stick to surfaces and may be more difficult to work with in subsequent steps of this protocol.

13. Place culture dish containing embryos into a 37°C 5% CO_2 incubator.

Embryo mounting and imaging

Timing: 10–15 h

Embryos are mounted onto an optical plate in individual drops of culture media and live imaged by confocal microscopy.

14. Turn on microscope, camera, light source, and stage incubator.
15. Prepare the embryo imaging plate by arranging drops of ~0.2 μL KSOM onto the imaging plate. The number of drops should be equal to the number of embryos that will be imaged in the experiment (Figures 4A and 4B).

Note: Work quickly during this step as the volume of individual drops is small enough that they can evaporate if left to sit.

Note: Large drops should be avoided. Embryos are more likely to move around during the live imaging in larger drops and the drops themselves can detach from the plate, resulting in loss of data.

16. Cover the drops with mineral oil and place the plate in a 37°C 5% CO_2 incubator for at least 30 min to allow KSOM to equilibrate.
17. Mount embryos for imaging by transferring one embryo to each drop of the imaging plate.

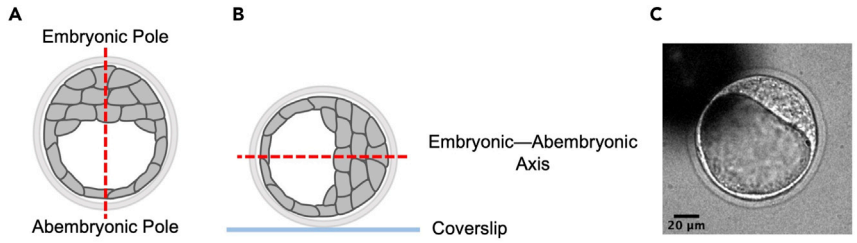

Figure 5. Blastocyst imaging orientation

(A) Schematic of blastocyst with embryonic-abembryonic axis in dashed red line.

(B) Schematic of ideal blastocyst orientation for imaging on a coverslip.

(C) Example bright-field image of a blastocyst.

⚠ CRITICAL: Position embryos such that the embryonic-abembryonic axis (Gardner, 1997) of the blastocyst runs parallel to the plane of the coverslip (Figures 5A–5C). This will be instrumental for the alignment and re-imaging section of the protocol.

18. Set imaging plate onto the microscope stage. Perform imaging according to biosensors/fluorescent reporters used in experiment (troubleshooting 2 and 3).

Note: Conditions will have to be optimized according to different experimental parameters and individual microscope assemblies. The following conditions were determined during optimization of live imaging from the original study, which included embryos expressing ERK-KTR-mClover and H2B-mRuby2 imaged in the YFP and mCherry channels (Pokrass et al., 2020).

a. Exposure Time: 200–250 ms
b. Laser Power: 22%–25% of maximum
c. Z-stacks: 5 µm steps
d. Imaging Interval: 15 min

19. 1 h before end of the imaging interval, continue to step 20.

Prepare 96-well round bottom plate for immunofluorescence

Timing: 30 min

A 96-well round bottom plate is coated with 1% agar and 0.9% NaCl to prevent embryos from sticking to surfaces during the immunofluorescence staining.

20. Prepare plate coating solution by microwaving 10 mL molecular biology-grade water with 1% agar and 0.9% NaCl until dissolved.
21. Pipette 50 µL coating solution to wells of a 96-well round bottom plate.
22. Gently tap plate on bench top to settle solution to the bottom of all wells.
23. Cover plate with lid or aluminum foil and set in 4°C refrigerator. The plate can sit for several hours but should be used within 24 h of its preparation.

Embryo retrieval and immunofluorescence

Timing: 1–2 d

Following live imaging, embryos are fixed and immunolabeled for lineage markers. This immunofluorescence protocol is performed in a 96-well plate format in which embryos are serially passed from left-to-right into wells containing the appropriate solutions.

Table 1. Buffers to add to immunofluorescence plate

Column	Solution
1	4% formaldehyde
2	PBX
3	Permeabilization Buffer
4	PBX
5	Blocking Buffer
6	Primary Antibody in Blocking Buffer
7	PBX
8	PBX
9	PBX
10	Blocking Buffer
11	Secondary Antibody in Blocking Buffer
12	n/a

Immunofluorescence Plate

⚠ CRITICAL: Fixing embryos *immediately* at the end of the live imaging period is essential for *in silico* alignment of embryos from the live imaging and endpoint datasets.

Note: The H2B-mRuby2 signal from ERK-KTR embryos is retained during fixation and can be used as the nuclear marker for both live and immunofluorescence imaging.

Note: This method was learned from the Hadjantonakis lab and adapted from previously published protocols (Frankenberg et al., 2013).

24. Thaw 1 mL of 4% formaldehyde in PBS.

⚠ CRITICAL: Quality of formaldehyde solution is crucial for fixation. 4% formaldehyde can be stored for up to 1 year at −20°C. Use aliquots thawed the day of the experiment for best results.

25. While formaldehyde thaws, label the coated 96-well round bottom plate according to Table 1.
26. Fill columns 1–6 with solutions indicated in Table 1. That is, pipet 100 μL 4% formaldehyde to column 1, 100 μL PBX to column 2, and so on.

Note: You will need as many rows as you have embryos in the experiment. Experiments with >8 embryos will accordingly need additional 96 well plates.

27. Immediately at the end of the imaging time course, bring the imaging plate and immunofluorescence plate to the dissection microscope.
28. Transfer embryos from the imaging plate into column 1 of the immunofluorescence plate containing 4% formaldehyde at a ratio of one embryo per well. Incubate for 10 min at 20°C–25°C.

Note: It is important to keep track of individual embryos. A simple numbering system is effective.

29. Wash embryos once by passing them into PBX.

Pause point: Fixed embryos can be stored for up to a week at 4°C protected from light. Transfer fixed embryos to a well with 4% BSA in PBS and cover with mineral oil to prevent evaporation.

Table 2. Buffers to add to final wash plate

Column	Solution
1	PBX
2	PBX
3	PBS
4	n/a
5	n/a
6	n/a
7	n/a
8	n/a
9	n/a
10	n/a
11	n/a
12	n/a
Final Wash Plate	

30. Incubate embryos in permeabilization buffer for 5 min at 20°C–25°C.
31. Wash embryos once in PBX.
32. Block 1 h at 20°C–25°C.
33. Incubate in primary antibody diluted in blocking buffer at least 16 h at 4°C. Cover wells containing primary antibody with mineral oil to prevent evaporation and cover plate with lid or aluminum foil.

Note: Primary antibodies were diluted as follows: Rabbit anti-NANOG (1:500), Goat anti-GATA6 (1:2,000), Mouse anti-CDX2 (1:100). Antibody dilutions should be optimized according to individual experimental parameters.

34. On the following day, fill columns 7–11 with solutions according to Table 1.
35. Wash embryos three times in PBX.
36. Block 1 h at 20°C–25°C.
37. Incubate embryos in secondary antibody diluted in blocking buffer for 60–90 min at 20°C–25°C. Cover wells with mineral oil and protect from light.

Note: Secondary antibodies were diluted as follows: Donkey anti-Rabbit (1:400), Donkey anti-Goat (1:1,000), Donkey anti-Mouse (1:1,000).

38. During secondary antibody incubation, prepare a final wash plate. Add 50 uL plate coating solution to columns 1–3 of a fresh 96-well round bottom plate. Put at 4°C and add solutions according to Table 2 after plate coating solution has solidified.
39. Wash embryos twice in PBX.
40. After the last wash, leave embryos in PBX and prepare imaging plate as in step 15; however, using PBS instead of KSOM for the mounting medium.
41. Wash embryos once in PBS, then transfer to imaging plate.

Note: Careful pipetting the embryos. They can easily get stuck to glass when transferred to solutions without detergent like PBS. Using a pipette that has previously held PBX can prevent embryo loss.

Note: To the extent that is possible, arrange embryos on the imaging plate in the same position and orientation they were in for the live imaging.

42. Perform imaging according to experimental protocol (Troubleshooting 4).

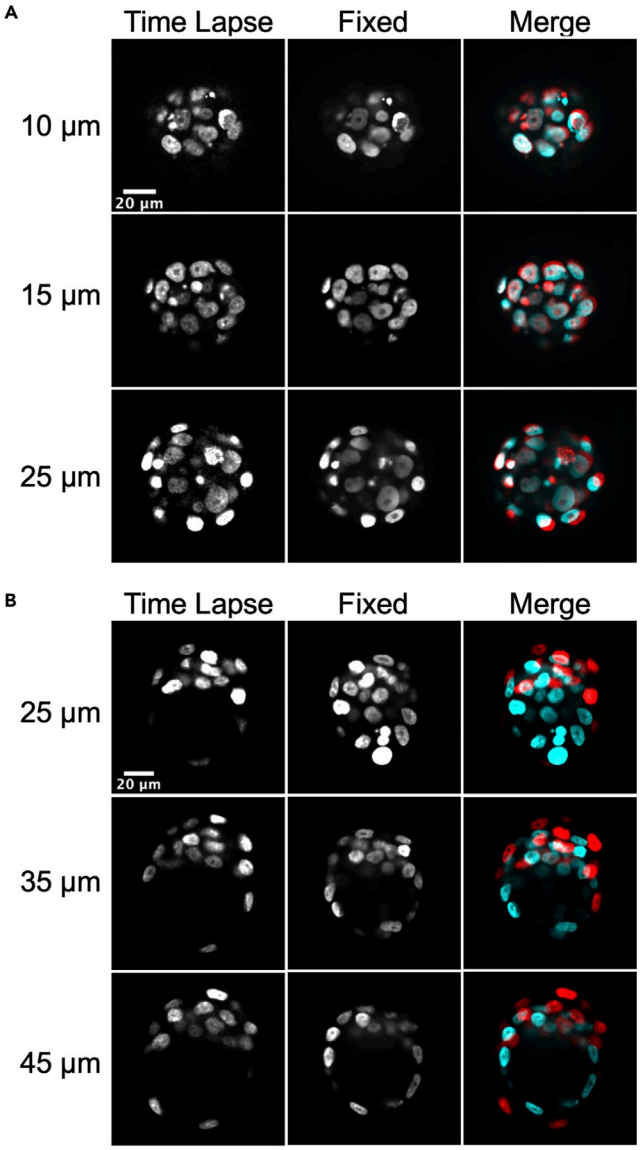

Figure 6. *In silico* alignment examples

Images were collected as described in step-by-step methods and inspected for nuclear overlap. 3 planes of a representative blastocyst are shown at different sample depths from both the time-lapse and immunofluorescence data. Optical section labels correspond to sample depth in the time-lapse image stacks.

(A) Example of good alignment.

(B) Example of poor alignment.

Alignment inspection and embryo re-imaging

Timing: 2–6 h

43. Using an image processing program like Fiji (Schindelin et al., 2012), load the nuclear marker images representing the last frame of the time-lapse imaging and the fixed images of each embryo.
44. Examine both image stacks closely for each embryo and determine if they can be aligned by eye (Figures 6A and 6B). Good alignment represents the agreement between both datasets such

that the same cells can be unambiguously identified based of off morphological and cytological features in the live and fixed image stacks. [Troubleshooting 5]

Note: If using an H2B nuclear marker, it can be helpful to use the nucleolus precursor bodies (Kyogoku et al., 2014) as landmarks within individual nuclei.

Note: Rotating image sets in the XY dimension can aid the visual determination of alignment.

45. Record which embryos failed to align.
46. Take the imaging plate back to the dissection microscope. For any embryos that could not be aligned, gently reposition them using a mouth pipette. It is best to rotate the blastocysts along the embryonic-abembryonic axis.
47. Re-image the embryos manipulated in the previous step. Check alignment and repeat as necessary until images that can be used for alignment are obtained.

Note: Pay attention to photobleaching in re-imaged embryos. Try to keep the total number of times any embryo is imaged to three or fewer.

48. Once images suitable for alignment have been obtained, proceed with analysis of time-lapse and fixed imaging data.

Image analysis

Timing: 1–3 h per embryo

49. Load time-lapse and immunofluorescence image data in Fiji as stacks.
50. Using the nuclear marker channel in each stack, perform any image translation or rotation required to identify corresponding cells in each data set (Figure 7A). It may be helpful to crop the images.
51. Duplicate the nuclear marker channel of the immunofluorescence image stack (Figure 7B). This will serve as a record of which cells have been analyzed.
52. Using the multi-point tool, give all cells that will be analyzed a number on the duplicate nuclear marker stack (Figure 7C).
53. In the Fiji menu select "Analyze -> Set Measurements…" to preset the parameters that will be recorded by the measure command.

Note: Area, mean value, centroid, and stack position are helpful to have in most experiments.

54. Begin analyzing cell 1 (from the numbering in step 48) on the time-lapse stack. Starting from the last frame of the experiment, work backwards to identify the starting position of the cell. If using the ERK-KTR, measure independent intensities for the cell's nucleus and cytoplasm for each time point of interest in the experiment by drawing regions of interest (Figure 7D).

Note: Use the nuclear marker signal to identify the nucleus and cytoplasm for each cell. Make measurements in the plane where the nucleus has the greatest diameter and do not exclude nucleolar areas in the ROI. Individual blastomeres have small cytoplasmic compartments. Measuring a narrow region that immediately borders the nucleus is recommended to avoid unintentionally measuring signal from neighboring cells.

55. Continue analyzing the remaining cells in the time-lapse stack. Save the measurements in your preferred spreadsheet software.

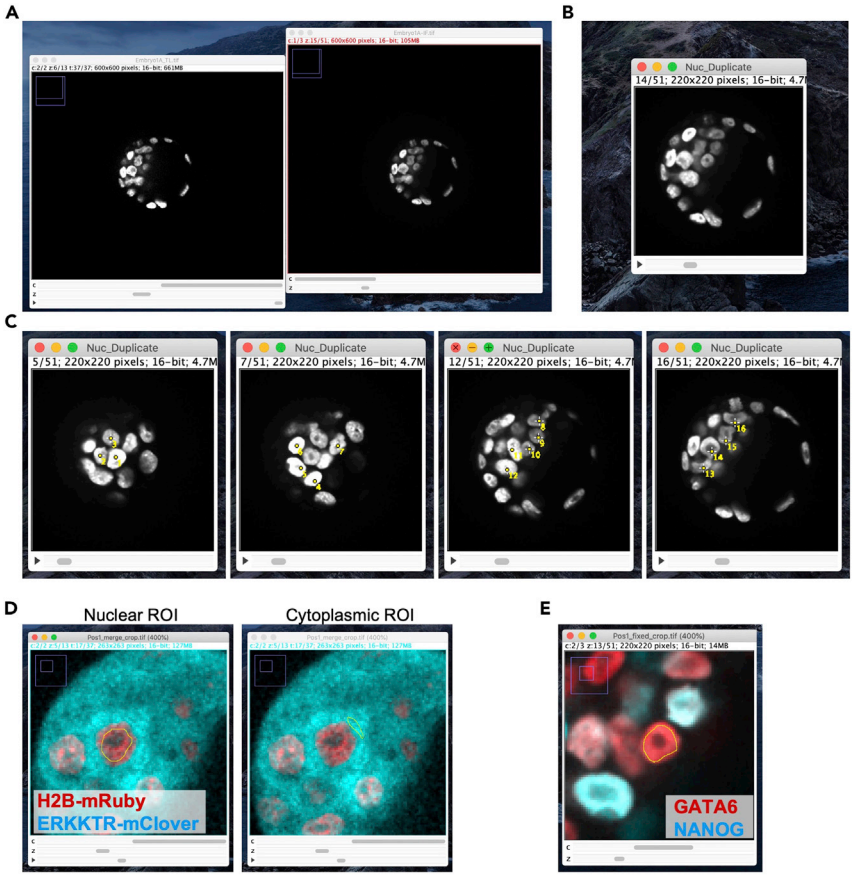

Figure 7. Image analysis in Fiji

(A) Time-lapse and immunofluorescence images as stacks.

(B) Duplication of the nuclear marker channel from the immunofluorescence image stack.

(C) Several planes of the duplicate stack in (B) with multi-points to indicate cell number in analysis.

(D) Example ROIs to analyze KTR intensity in the nucleus and cytoplasm. ERK-KTR (cyan) and H2B (red) have been merged to facilitate identification of nuclear and cytoplasmic cell compartments.

(E) Example ROI to analyze immunofluorescence signal in fixed cells. Overlay of GATA6 (red) and NANOG (cyan) is shown.

56. After completing measurements from the time-lapse data, measure nuclear intensities of the lineage markers stained for in the immunofluorescence protocol by drawing nuclear ROIs. Measure cells according to the same numbering convention as in step 50 (Fig 7E).
57. Save the immunofluorescence data analysis and proceed with analyzing remaining embryos from the experiment.

EXPECTED OUTCOMES

Females from the mouse strain referred to in Pokrass et al. (2020) generally had 6–10 embryos on the day of the dissection. Genetic background, strain, animal age, and other factors can all influence the number of embryos per successful mating attempt (Rugh, 1990). Mouse breeding pairs do not always successfully mate the day the cages are set and there is not an easy and reliable test to determine if the females used in the experiment are pregnant before sacrificing the animals. Checking mice for vaginal plugs can aid in the identification of pregnant females, but the reliability of this indicator varies among mouse strains (Mader et al., 2009). Developing a breeding scheme in which 3–4 potentially pregnant females can be sacrificed on the day of the experiment can maximize the chances to get one or more litters of blastocysts.

Biosensor fluorescence intensity will vary depending on multiple factors including the promoter, genomic integration site, fluorophore, and protein stability (Dobie et al., 1996; Haruyama et al., 2009; Palmer et al., 2011). In addition to transgene design, the mouse breeding scheme can be optimized for reporter expression. For instance, mating males and females that are homozygous for the fluorescent reporter allele can maximize both the number of transgenic embryos and the expression levels in the litter. Moreover, zygotic expression of the transgene from maternally derived mRNAs (Li et al., 2010) can further enhance reporter signal intensity and may be particularly important for experiments that require analysis of young blastocysts (~E3.25).

Embryo manipulation is a technically challenging skill and it is not uncommon to lose samples at any stage of the experiment. The immunofluorescence protocol in particular, wherein embryos must be individually passed into several different solutions is particularly difficult to accomplish without loss of embryos. With patience and practice, however, users will become more proficient and the rate of embryo loss will decline.

LIMITATIONS

We expect this protocol to be compatible with multiple genetically encoded biosensors for quantifying signaling pathway activity; however, the *in silico* alignment step required to connect live cell measurements with endpoint analysis depends on the presence of a nuclear marker for success.

While blastocysts tolerate stresses such as low temperatures and shear force, they are particularly sensitive to phototoxicity (Xenopoulos et al., 2012). This protocol may be unreliable when imaging fluorophores that are particularly dim (and therefore require more intense laser power and longer exposure times) or require ultraviolet exposure of live embryos. To this end, the use of live-cell nucleic acid stains that occupy the DAPI channel are not recommended and should be substituted with genetic labeling methods.

Because of the positioning of the ICM and blastocoel cavity, blastocysts have a morphological polarity that aids the *in silico* alignment step (Gardner, 1997). Pre-cavitation embryos – lacking this polarity – may be incompatible with this protocol when attempting to connect the live and fixed cell measurements.

TROUBLESHOOTING

Problem 1

Mouse mating pairs do not generate pregnant females (step 10)

Potential solution

Mouse mating behavior varies among strains and with social and environmental factors (Palanza et al., 2005). Identification of animals in late pregnancy is possible by visual observation, manual palpation, or measurement of mass increase, but determining early pregnancy is much more difficult (Mader et al., 2009). If the majority of mice sacrificed for experiments are not pregnant or have younger than expected embryos it can be helpful to adjust the mating strategy to increase the number of successful breeding attempts.

The mouse estrous cycle, divided into the proestrus, estrus, metestrus, and diestrus phases, repeats every 4–5 days. Selecting females in proestrus or estrus can increase the likelihood that mating will occur and lead to fertilization. Visual inspection of the genitalia is the simplest way to identify animals in proestrus or estrus, but strain and coat color differences can cause confusion when first learning this method (Detailed explanation and reference images in (Byers et al., 2012)). The vaginal cytology method, which involves swabbing the mouse vaginal wall, staining the cells, and viewing them under a microscope, is more accurate but also requires more time and reagents (Byers et al., 2012).

The observation of a vaginal plug the morning after setting up a mouse breeding pair is a common method to determine which animals mated overnight; however, plug and pregnancy rates, and the reliability of a vaginal plug as an indicator of pregnancy, vary among mouse strains (Mader et al., 2009). Keeping a record of which mice were plugged and which became pregnant can generate data to assist the design of a mating strategy that maximizes successful breeding attempts while minimizing the total number of mice needed for experiments. If mice only become pregnant following an observable plug, exclusively sacrifice plugged females to obtain embryos. However, if many mice become pregnant without the presence of a plug, it may be necessary to sacrifice females after mating attempts regardless of plug observation. In this case it is particularly important to select animals in the proestrus and estrus phases.

Problem 2

Embryo death during live imaging (step 18)

Potential solution

The most likely reason that embryos die during the live imaging is phototoxicity; however, death can also reflect an issue with the culture conditions. To distinguish these possibilities, include two non-imaged controls in experiments: mounted and unmounted. The mounted control consists of embryos that are mounted to the imaging plate in step 17 but are not included in the subsequent live imaging step. The unmounted control consists of embryos that are left to remain on the embryo culture plate in the incubator during the live imaging.

If the imaged embryos die but the mounted and unmounted controls survive, the laser power settings and exposure times of the experimental protocol need to be reduced. If the imaged embryos and mounted controls die but the unmounted controls live, this could reflect a failure of the microscope stage environmental control chamber or some insult that occurred during the mounting step. If embryos across all conditions die, the embryo culture reagents should be tested against validated stocks. The mineral oil used to cover KSOM is often the reason for death in this case, but all reagents should be tested to determine if any are the cause.

Problem 3

Excessive embryo tumbling during live imaging (step 18)

Potential solution

Embryo tumbling tends to result from movement of the microscope stage and convection currents in the imaging media. To minimize the total distance the stage must move during live imaging, arrange the drops of KSOM on the imaging plate as close together as possible without having two drops merge together. Limit the volume of media in each drop as well, as this can both allow a tighter arrangement of drops on the imaging plate and reduce the chance of convection currents. Ideally, individual drops should be about 5 times the diameter of the average blastocyst. If possible, adjust the stage movement speed in the XY dimension to the slowest possible setting to prevent jostling embryos during image acquisition.

Convection in the KSOM drops can arise as a result of the microscope live imaging set up. For example, using an oil-immersion objective to image embryos could introduce recurring temperature fluctuations as the room temperature objective cycles through positions on the heated imaging plate. In this case using an objective heater could alleviate the issue.

Problem 4

Poor immunofluorescence signal (step 42)

Potential solution

The zona pellucida (ZP) is a glycoprotein matrix that envelops the zygote from the single cell stage until the eventual hatching of the blastocyst (Wassarman, 2008). Different primary antibodies used for immunofluorescence show different degrees of nonspecific binding to the ZP. Antibodies which tightly bind the ZP may fail to bind their intended epitopes, resulting in weak or no signal in the expected localization patterns. A bright signal in the ZP itself can serve as an indication that the primary antibody used is binding to this structure.

Removal of the ZP can improve immunofluorescence signal and is a standard practice in preimplantation development labs. There are multiple methods for ZP removal, but the most common is by gentle acid wash (for detailed method see Nagy, 2006). If performing ZP removal, be mindful that embryos will become more likely to stick to surfaces, particularly plastic ones, and this could present challenges in processing embryos through both the live imaging and immunofluorescence sections of the protocol.

Problem 5

Poor alignment between live and fixed imaging data sets (step 44)

Potential solution

It is very likely that *in silico* alignment will not be possible for >50% of embryos in an experiment after the first round of imaging. The re-imaging steps are critical to increase the number of samples that can be analyzed in each experiment; however, this section of the protocol can be technically challenging. It is possible that inexperienced users will not have positioned the embryos correctly at either the live or fixed imaging step. Examining the nuclear marker images will provide a clue if this is the case. Practice positioning embryos with the embryonic-abembryonic axis parallel to the plane of the coverslip. Alternatively, some embryos can become significantly deformed during the immunofluorescence protocol and will not be suitable for alignment despite re-imaging attempts.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sergi Regot (sregot@jhmi.edu).

Materials availability

The ERK-KTR^{LSL} mice generated in Pokrass et al. 2020 have been deposited to the Jackson Laboratory as JAX#035566.

Data and code availability

Live imaging of embryos and corresponding fixed embryo data generated in Pokrass et al. (2020) are available at the BioImage Archive (Accession S-BIAD28).

ACKNOWLEDGMENTS

We thank all members of the Regot lab for helpful discussions and technical advice. We thank the Nathans lab and the JHSOM Transgenic Animal Core Facility for training on the handling of mice and collection of mouse embryos. We thank the Hadjantonakis lab for training on the manipulation and immunostaining of mouse blastocysts. We acknowledge our funding sources: an NIH T32 predoctoral training grant and NSF Graduate Research Fellowship to M.J.P. (DGE-1746891), an NSF CAREER award (MCB-1844994), NIGMS R35 (1R35GM133499), American Cancer Society Research Scholar Grant (133537-RSG-19-005-01-CCG), and Jerome L. Greene Foundation Discovery Award to S.R.

AUTHOR CONTRIBUTIONS

M.J.P. and S.R. conceived and optimized the method. M.P. wrote the manuscript and prepared demonstrative figures. S.R. supervised the study and secured funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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