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Method Article

Histological cut of a paraffin-embedded blastocyst: Optimized protocol for murine blastocysts

Alejandra Usón Gracia[a], Marta López-Morató[a], José Mijares[a], Soledad Sánchez-Mateos[a], Francisco Miguel Sánchez-Margallo[a], Ignacio Santiago Álvarez[b], Nuria Hernández[a,*]

[a] *Jesús Usón Minimally Invasive Surgery Centre, Cáceres, Spain*
[b] *Universidad de Extremadura, Badajoz, Spain*

A B S T R A C T

Paraffin-embedded tissues have been used for research and therapeutic applications for decades, as they represent a valuable tool in histology and for molecular analysis, as well as being a way to preserve tissue samples for long periods at a low cost. For tissues such as the liver, lungs, kidney, heart or brain, there are many protocols available, already optimized. The purpose of this work is to optimize and simplify the protocols already available to take a single blastocyst from a mouse, fix it and embed it into a paraffin block without using gelatin, to then perform histological cuts using a microtome, with no need of sophisticated equipment or trained personnel.

- The protocol presented here preserves well the morphology of the blastocyst.
- Paraffin-embedded sections of the sample can be used for studies such as *in situ* hybridization, immunohistochemistry, enzyme histochemistry, DNA, RNA or protein extractions, analysis of biomarkers, characterization of surface markers of stem cells integrated into the embryo, to prepare histological material for educational purposes, etc.
- Some of these studies could represent a valuable source of new information for the field of reproductive biology.

A R T I C L E I N F O

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* Corresponding author.
E-mail addresses: ausongracia@gmail.com (A.U. Gracia), mlopez@ccmijesususon.c (M. López-Morató), jmijares@ccmijesususon.com (J. Mijares), ssanchez@ccmijesususon.com (S. Sánchez-Mateos), msanchez@ccmijesususon.com (F.M. Sánchez-Margallo), ialvarez@unex.es (I.S. Álvarez), nhernandez@ccmijesususon.com (N. Hernández).

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Resource availability:	Not applicable.

Method details

The information currently available to embed blastocysts into paraffin blocks include: protocols using gelatin and paraffin [1,2], protocols to embed a piece of uterus containing the blastocyst already implanted [3,4], and a few protocols for *Xenopus* or bovine embryos, using specific equipment that might not be available in every laboratory [5,6]. Nonetheless, little information is available on easy protocols to embed single mouse blastocysts into a paraffin block to then make histological cuts. Furthermore, this protocol would represent an advantage over the available protocols that use gelatin and paraffin, due to the fact that the gelatin is soft and is not hardened into the paraffin block, so the blastocyst could be damaged when histological cuts are performed.

The following protocol describes the embedding of mice blastocysts, obtained from the uterus of a pregnant female, into a paraffin block in order to make histological cuts of the sample. The main steps of the protocol are fixation, permeabilization, staining, dehydration and embedding of the sample, the blastocyst, into the paraffin block. The blastocyst can then be preserved in paraffin for a long period of time, or it can be cut using a microtome for additional study of its morphology, biological markers, etc. The next sections describe the materials and reagents used for this purpose, and the detailed protocol along with tips for troubleshooting.

Materials and reagents

- Mice blastocyst (additional media and equipment needed depending on the methods employed to obtain the blastocysts)
- 4 % Paraformaldehyde (PFA), freshly prepared (Sigma)
- Polyvinylpyrrolidone (PVP) (Sigma)
- 1X Phosphate-Buffered Saline (PBS), pH 7.2 (Sigma)
- Fetal Bovine Serum (FBS) (Sigma)
- Triton X-100 (Sigma)
- Xylene
- Ethanol 96 % and 100 %
- Centre well dish (EMB)
- 4-well dish (EMB)
- Pasteur pipette
- P10 pipette
- Stripper pipette (used to handle oocytes and embryos)
- Stripper capillaries 135μm
- Pipette tips (Eppendorf)
- Paraffin wax
- Paraffin dispenser – Leica EG1150C
- Microtome Minot – Leica RM2255
- Embedding molds and rings
- Tweezers
- Fine brushes

- Section warmer set at 35 °C
- Hematoxylin
- Eosin
- Microscope
- Microscope lamp
- Cover slides (Thermo Fisher Scientific)
- Slides, albumin-treated (Thermo Fisher Scientific)
- Incubator
- DPX mounting media (Sigma)
- Glass cuvettes used for staining, 105 × 85 × 70 mm

Method workflow

All animal experiments were approved by the local ethics committee.

Collection of blastocysts from mice

Blastocysts can be obtained via different ways. Briefly, for this experiment, B6D2 strain female mice were hormonally stimulated with 10 IU of eCG, followed by a stimulation of 10 IU of hCG 48 h later, and mated with the male. Four days later, the females were euthanized and each uterus was retrieved by performing an abdominal incision. Each uterus with the two horns was placed in M2 media pre-warmed at 37 °C and flushed with an insulin syringe. Blastocysts were then retrieved using a stripper pipette and transferred to a fresh dish containing KSOM media until further processing.

Fixation and permeabilization of the blastocyst

1 To fix the blastocyst, transfer it from KSOM media to a centre well dish with 500 μl of 4 % PFA with 0.02 % PVP. Leave the blastocyst in 4 % PFA + 0.02 % PVP for 30 min at room temperature (RT) or for 24 h at 4 °C.

Tip: A good fixation is that which preserves and maintains the morphology of the sample in as life- like state as possible, as well as gives a good signal-to-noise ratio after in situ hybridization. There are many fixatives available, but here to fix the embryos, freshly prepared 4 % PFA is essential for good sample fixation.

Tip: PVP, or also polyvinyl-alcohol (PVA), can be used to replace serum or BSA. It is aimed at counteracting stickiness in the absence of albumin when protein-free medium is used, to prevent adhesion of the embryos to the bottom of the dish [7].

2 Wash the blastocyst in a 4-well dish with 500 μl of PBS with 5 % FBS (fetal bovine serum), for 1 min at RT, to remove the remaining PFA.

Tip: As previously mentioned for the PVP, FBS is used in this case to prevent adhesion of the blastocysts to the bottom of the dish.

3 Permeabilize the zona pellucida of the blastocyst by keeping it for 20 min at RT in a 4-well dish with 500 μl of Triton X-100 (0.2 % of Triton X-100 diluted in PBS with 0.02 % PVP).

Tip: Triton X-100 is a nonionic detergent widely used to permeabilize the membrane of the cells. In our case the use of Triton X-100 was crucial to permeabilize the zona pellucida of the blastocyst so that the paraffin can properly enter the blastocyst. In the absence of this step, an inadequate embedding of the blastocyst was achieved.

Tip: We do not recommend the use of blastocysts lacking the zona pellucida. Even though these are dehydrated and permeabilized quicker, when histological cuts are performed, the microtome seems to disrupt the blastocyst and we could only obtain small pieces of part of the trophoblast.

4 Wash the blastocyst in a 4-well dish with 500 μl of PBS with 5 % FBS (fetal bovine serum), for 1 min at RT, to remove the remaining Triton X-100.

Staining and dehydration of the blastocyst

Due to the small size of the mouse blastocyst, to ease the remaining steps we decided to stain the blastocyst to be able to detect it more easily.

5 Using a Pasteur pipette, place a drop of 2 % eosin on a slide. Take the blastocyst using a pipette, place it in the eosin drop and leave it for 15 s to stain the sample (Fig. 1). Alternatively, this can be performed on a 4-well dish.

Tip: This step can be performed by visualizing the blastocyst under a microscope. Once the sample is stained, it will have a pink-ish tone and it will be easier to visualize in the next steps, as one of the problems with this protocol is the loss of the blastocyst when reagents that change densities are used.

6 Wash the blastocyst in a 4-well dish with 500 μl of PBS with 5 % FBS (fetal bovine serum), for 1 min at RT, to remove the remaining eosin.

7 Dehydrate the blastocyst using ethanol at different concentrations. Transfer the blastocyst to a 4-well dish containing 96 % ethanol. Incubate for 10 min and transfer to another well containing 100 % ethanol. Incubate again for 10 min to completely dehydrate the sample.

Tip: Shorter incubation times can lead to incomplete dehydration, rendering unusable paraffin blocks.

8 Transfer the blastocyst from the 4-well dish to a slide containing a few drops of xylene and leave for 5 min at room temperature.

Tip: This is a crucial step that must be carried out under the microscope in a hood, as due to the change of density between the ethanol and the xylene, the blastocyst can be easily lost in this liquid. Previous staining of the sample with eosin will help locate the blastocyst more easily.

Tip: The ethanol will dry the xylene and can cause the loss of the blastocyst. When transferring the blastocyst to the xylene from the previous step, take as little ethanol as possible to avoid this.

CAUTION: Xylenes are toxic organic solvents, so they must be handled in a hood. Furthermore, do not perform this step in a plastic dish, as the xylene will weaken the plastic and might even dissolve it.

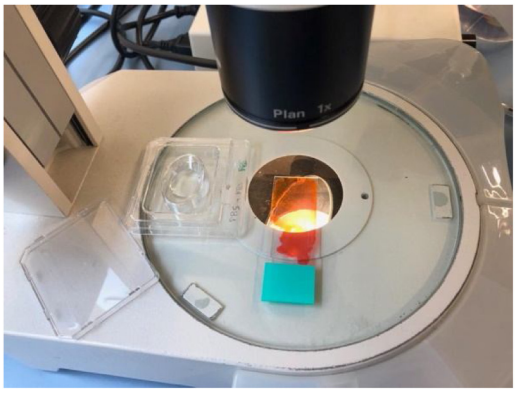

Fig. 1. Slide with a few drops of eosin, in which the blastocyst was stained for 15 s.

Embedding of the blastocyst into the paraffin block

9 Heat the paraffin at 70 °C and, using a paraffin dispenser, fill an embedding mold until the top (Fig. 2). Immediately, using a pipette, recover the blastocyst from the xylene and place it inside the paraffin, at the bottom of the embedding mold.

Tip: When recovering the blastocyst from the xylene, take as little xylene as possible, as too much of it will destroy the paraffin and prevent formation of a proper paraffin block.

Tip: Care should be taken when cutting the paraffin block, as the blastocyst is difficult to visualize and can be located anywhere in the block.

10 Place an embedding ring properly labelled on top of the mold and fill it with melted paraffin. Leave to cool and harden overnight.

Tip: The embedding ring is used to attach the paraffin block to the microtome holding clamp. A correct labelling will facilitate future identification of samples.

Sectioning of the paraffin block

11 Place the paraffin block in a cold plate (between 10–15 °C) until the paraffin block is completely hardened and it has a white-grey color.
12 Attach the paraffin block to the holding clamp of the microtome and begin sectioning. Cut 6 μm sections. The blastocyst should be easily visualized due to the eosin staining.

Tip: Thicker sections are not recommended, as the blastocyst has a very small size and the desired cut may not be obtained.

13 Transfer the sections to a section warmer, filled with water at 35 °C, using a fine brush and tweezers. Leave until the paraffin sections are completely stretched (Fig. 3).

Tip: Heat stretches the sections and removes wrinkles. Temperature can be increased up to 45–50 °C, but do not leave slides on the water bath longer than necessary or the sections could be destroyed.
14 Mount each section on an albumin-treated slide properly labelled.

Tip: Conventional slides can be used, however it is preferred the use of albumin-treated slides, as the albumin treatment will prevent the paraffin section from detaching in the downstream washing steps.

Fig. 2. Paraffin dispenser and embedding mold.

Fig. 3. Water bath set at 35 °C to stretch the paraffin sections.

Tip: If desired, several samples can be mounted on the same slide. Do not place sections too close to the edge though.

15 The sample can be preserved until further use or can be immediately processed for analysis.

Processing and rehydration of the sample

16 Incubate the slide with the section in an incubator at 70 °C for 10 min.

Tip: Paraffin has a melting point between 46 and 68 °C. This step will melt the paraffin for its future elimination with xylene or the selected method.

17 Immerse the slide in a cuvette containing a volume of xylene that covers the slide, during 20 min to remove the paraffin.

Tip: This step can be repeated 2–3 times until the sample is completely free of paraffin.

18 Rehydrate the sample by immersing it in ethanol at different concentrations. Start with an immersion in 100 % ethanol for 10 min. Then immerse the sample in 96 % ethanol for 10 min.

19 Stain the sample by immersing it in a cuvette containing hematoxylin for 11 min.

Tip: Hematoxylin is a basic deep-purple dye that stains basophilic substances such as DNA or RNA. In this case the hematoxylin will stain the nucleus of the cells with a blue color.

Tip: Longer staining times cause overstaining of the nuclei, being difficult to properly see the nuclear details. Shorter staining times produce an insufficient staining of the blastocyst.

20 Wash the sample carefully with tap water to remove the excess of hematoxylin.

Tip: Avoid pouring the water directly onto the sample, as this may cause detachment of the sample from the slide.

21 Stain the sample with eosin to stain the rest of the cellular structures. Immerse the slide in a cuvette containing eosin for 15 s and quickly wash the sample to avoid an overstaining.

Tip: Eosin is an acidic dye, negatively charged, that stains basic structures with a pink color. In this case, eosin is used to stain the cytoplasm of the blastocyst.

Tip: Do not increase the staining time, as an eosin overstaining can mask the hematoxylin staining.

Tip: As the eosin is more soluble in alcohol than in water, the sample needs to be dehydrated with different concentrations of ethanol to achieve a better staining of the cellular structures.

22 Dehydrate the sample again using different concentrations of ethanol. Start by immersing the sample in 96 % ethanol for 30 s and a final immersion in 100 % ethanol for 5 min.

23 Transfer the slide to a cuvette containing xylene for 10 min. Do not leave the xylene to dry and add a few drops of DPX mounting media.

Tip: DPX mounting media is a mixture of distyrene (a polystyrene), a plasticizer (dibutylphthalate) and xylene. It is a colorless, synthetic resin mounting media that preserves stains and dries quickly.

24 Finally, place a cover slide on top of the sample, avoiding the formation of bubbles. The sample is now ready for visualization under the microscope and subsequent analysis.

Tip: The presence of bubbles can interfere with the visualization of the sample under the microscope. If necessary, gently press the cover slide to remove any bubble.

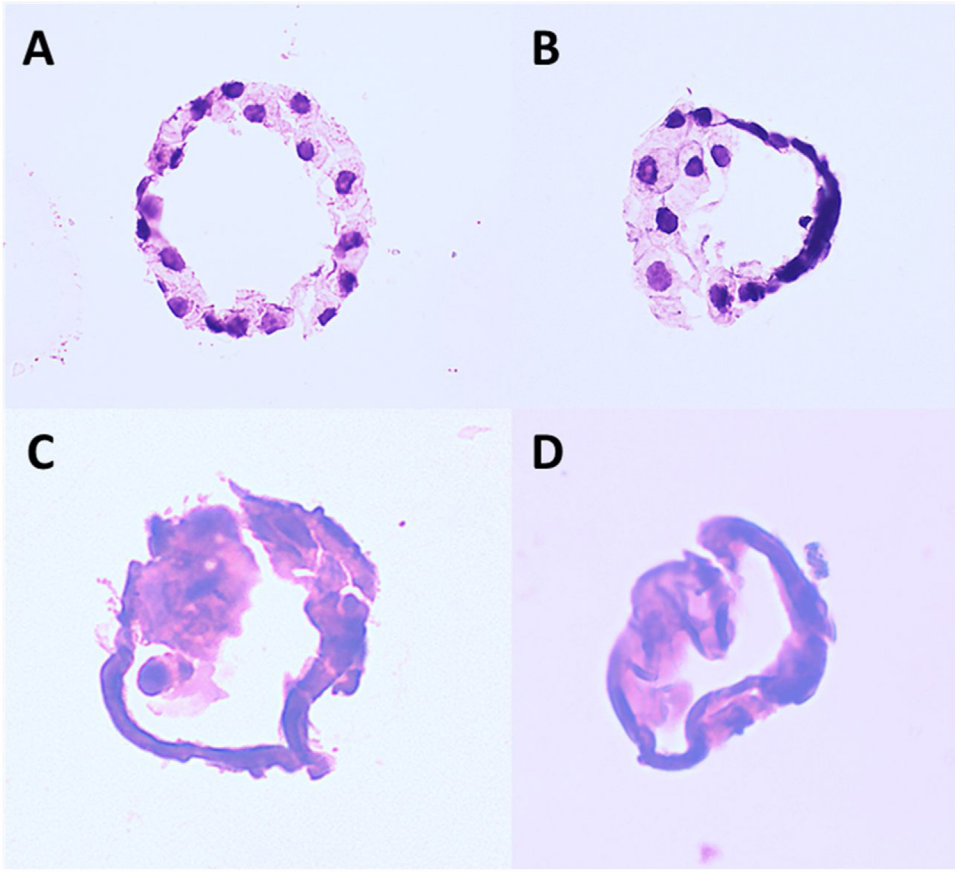

Fig. 4. Histological cuts of various paraffin-embedded blastocysts. A. Histological cut of a blastocyst with the morphology of the trophoblast well preserved. B. Histological cut of a blastocyst in which part of the trophoblast was well preserved, but half of it appears folded. C, D. Histological cuts of a blastocyst in which the cells of the trophoblast cannot be properly visualized.

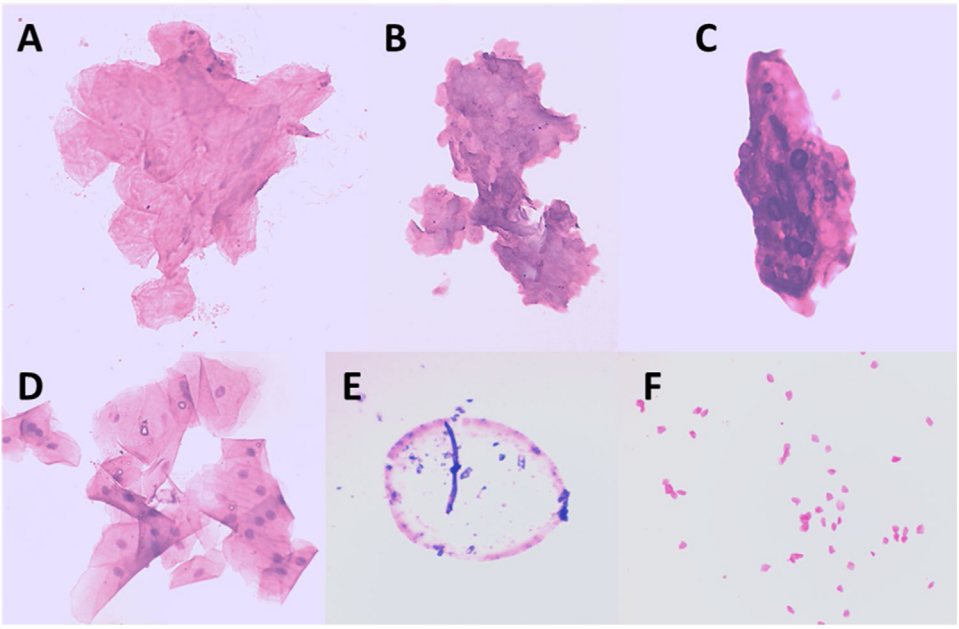

Fig. 5. Histological cuts of various paraffin-embedded blastocysts. A, B, D. Cells of part of the trophoblast of the blastocyst. C. Whole blastocyst folded onto itself. E. Histological cut of a blastocyst with the morphology of the trophoblast well preserved, but poorly stained, due to an insufficient time of staining. F. Disaggregation of cells of the blastocyst, probably caused by the microtome when the cut is performed.

Results

Several histological cuts of single blastocysts were performed during the optimization of the protocol described in this work. Once the protocol was optimised, we could obtain a complete sectioning of the blastocyst from 65 % of the blastocysts embedded. Fig. 4 shows the best histological cuts that could be obtained, based on the type of sample used and its properties. Fig. 4A shows a histological cut of a blastocyst with the morphology well preserved, whereas Fig. 4B–D show histological cuts of a blastocyst in which part of the sample was folded onto itself and not all the cells of the trophoblast can be properly visualized, although they retain a round-ish shape. The nucleus of the cells was stained with hematoxylin (blue) and the cytoplasm was stained with eosin (pink). Fig. 5 shows histological cuts of blastocysts in which the morphology of the sample could not be properly preserved and visualized in the paraffin section, due to different problems that are discussed below in the Troubleshooting section.

Conclusion

Here we have optimized a protocol to embed a single mouse blastocyst into a paraffin block and to perform histological cuts that preserve the morphology of the sample. The protocol has been optimized based on the results and problems faced during the realization of it. Nonetheless, it may need little adjustments depending on the conditions of each laboratory. Furthermore, it is worth mentioning that some problems may not be solved, as it is not possible to orientate the blastocyst when placed into the paraffin, it is difficult to visualize once embedded into the paraffin, so sections with part of the blastocyst that do not represent the expected morphology may be obtained, and the cut of the block is a critical step that can cause the blastocyst to fold onto itself or to break, due to the small size of the sample. However, we have demonstrated that using this protocol it is possible to obtain good quality sections of the blastocyst, in an easy, cheap and reproducible manner, which can

be used for subsequent analysis that could represent a new source of information for the reproductive biology field.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mex.2019.12.008.

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