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Fluorescent immunodetection of epigenetic modifications on pre-implantation mouse embryos

Contributors:

Claire Boulesteix^{1,2}, Nathalie Beaujean^{1,2*}

¹ INRA, UMR1198 Biologie du Développement et Reproduction, F-78350 Jouy-en-Josas, France

² ENVA, F-94700 Maisons Alfort, France

* **Corresponding author:** nathalie.beaujean@jouy.inra.fr

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Summary

A common problem in research laboratories that study the mammalian embryo after nuclear transfer is the limited supply of material. For this reason, new methods are continually developed, and existing methods for cells in culture are adapted to suit this peculiar experimental model. Among them is the fluorescent immuno-detection.

Fluorescent immuno-detection on fixed embryos is an invaluable technique to detect and locate proteins, especially nuclear ones such as modified histones, in single embryos thanks to its specificity and its sensitivity. Moreover, with specific fixation procedures that preserve the 3D shape of the embryos, immunostaining can now be performed on whole-mount embryos. Target proteins are detected by specific binding of first antibody usually non-fluorescent, and revealed with a second antibody conjugated with a fluorochrome directed specifically against the host animal in which the first antibody was produced. The result can then be observed on a microscope equipped with fluorescent detection. Here, we describe the 3D fluorescent immuno-detection of epigenetic modifications in mouse embryos. This procedure can be used on nuclear transferred embryos but also on in vivo collected, in vitro developed and in vitro fertilized ones.

1-Introduction

During normal development the two very specialized gametes, the sperm and the oocyte, undergo profound changes. They lose their characteristic nuclear and chromatin configuration giving way to the establishment of an embryo-specific chromatin and nuclear organization. It is exactly this distinctive embryonic configuration that should be achieved by the donor nucleus after nuclear transfer (NT) into an enucleated oocyte. This conversion of a differentiated nuclear organization into an embryonic one is forced by the intimate contact between the donor cell chromatin and the oocyte cytoplasm, allowing its “reprogramming”. Although the mechanisms of this reprogramming remain poorly understood, it appears that the particular chromatin landscape established after nuclear transfer is crucial for embryonic genome expression and for further development (1–4).

Whereas the genetic information provides the framework for the manufacture of RNAs and proteins, chromatin structure and nuclear architecture indeed control the accessibility of proteins to the DNA, especially transcription factors and RNA polymerase, and thereby gene expression (5). It involves several mechanisms such as epigenetic modifications (DNA methylation or histone post-translational modifications for example) that can be examined by immunostaining (6).

During the past decade, 3D immuno-detection on fixed NT embryo has therefore provided tremendous insight into the sub-nuclear positioning of proteins during these very first stages of development, and into mechanisms - such as the epigenetic ones modifying the degree of chromatin compaction - involved in the reprogramming of the donor cell (7).

Fluorescent immunostaining allows the simultaneous detection and localization of two or three epigenetic modifications by combining primary antibodies produced in different species, and secondary antibodies with different fluorochromes. Moreover, immuno-detection makes it possible to have results even with a single embryo at the 1-cell stage, thereby reducing the problem encountered by the limited availability of embryos after nuclear transfer.

The immunofluorescent-staining protocols usually used on thin cells in culture tend to flatten the specimens and are not optimal for the analysis of embryos. However by using alternative fixation procedures ensuring that the specimens maintain the 3D structure they had in vivo, 3D data sets can now be obtained on preimplantation embryos with a preserved shape (8,9).

By combining this technique with high resolution microscopy – Structured Illumination Microscope or Confocal Laser Scanning Microscope- and precise image analysis it is also possible to quantify the epigenetic modification of interest and then to describe its evolution not only in term of presence or absence, but also in terms of localization and quantity during pre-implantation development (4,10–13).

Here, we describe the 3D fluorescent immuno-detection of epigenetic modifications in mouse embryos with 1) the fixation of the embryos, 2) the immunostaining itself and 3) the observation procedure.

2-Materials

For all the procedures some basic equipment is required (**Fig. 1**).

1. Dissecting microscope with appropriate working distance
2. Heating block set at 27°C (*see Note 1*)
3. Aspirator tube: about 40 centimeters length (*see Note 2*) (**Fig. 2**)
4. Thin glass manipulation pipettes for embryos, usually home made in the flame with glass capillaries or Pasteur pipettes (*see Note 3*) (**Fig. 1**)
5. Embryo glass dishes with glass lids (*see Note 4*) (**Fig. 1**)

2.1 Embryo fixation

1. pH indicator strips
2. Fixative: 20% paraformaldehyde (PFA) solution. Upon opening of the stock solution, adjust pH to ~7.0 with 0.2N NaOH if necessary and store maximum 1 month at 4 °C with light protection (aluminum foil for example). Before each experiment pH should be checked with a pH indicator strip: if lower than 7.0 or upper to 7.4, through it away.
3. Phosphate Buffered Saline (PBS): prepare according to manufacturer's instructions, autoclave and store at 4°C. Filter through a 0.22-µm sterile cellulose acetate membrane before use.
4. Parafilm

2.2 Immunostaining

Prepare all solutions using ultrapure water (18.2 MΩ•cm resistivity at 25 °C).

1. Phosphate Buffered Saline (PBS): same preparation as above (step 3 in section 2.1)
2. Permeabilization stock solution: 10% Triton X-100 stock solution is prepared with ultrapure water (w/v) and mixed slowly to avoid bubbles. Store at 4 °C for maximum 1 month.
3. PBS-BSA 2% solution: dissolve embryo/culture tested BSA powder slowly in PBS (w/v) and filter through 0.22-µm sterile cellulose acetate membranes. Aliquots of 1mL or 2mL can be kept at -20°C for several months and thawed just before use. After thawing filter through a 0.22-µm sterile cellulose acetate membrane, store at 4 °C.
4. Primary and secondary antibodies (*see Note 5*)
5. Aluminum foil
6. Petri dishes (35 or 60 mm diameter)
7. 0.5 or 1.5mL micro test tubes
8. Mineral oil, embryo tested: keep at room temperature and protect from direct light.
9. 37°C oven or incubator (*see Note 6*)
10. Nucleic acid stain (*see Note 7*)
11. Mounting anti-fading medium
12. SuperFrost Plus slides
13. Glass cover slips (*see Note 8*)
14. Clear nail polish
15. Thin micro-spatula

2.3 Microscope observation

High resolution microscopy - such as Structured Illumination Microscope or Confocal Laser Scanning Microscope - is highly recommended.

3-Methods

3-1 Embryo fixation

1. Label the glass dishes and lids with appropriate signs in order to identify the different groups (control, NT etc...), date and size (n=...).
2. Choose between the two types of fixation:
 - a short one : 20 minutes at room temperature (22-25°C or 27°C on the heating plate) with 2% PFA freshly diluted in Phosphate Buffered Saline PBS, if you plan to perform immunostaining immediately after.
 - a long one : overnight at +4°C with 4% PFA, freshly diluted in Phosphate Buffered Saline PBS, if you plan to perform immunostaining the day after. If necessary keep the embryos in PFA for up to one week - in that case, change the PFA 4% every 2 days (*see Note 9*).
3. Prepare in each glass dish a volume of ~500µL PFA, diluted with PBS.
4. With a thin glass manipulation pipette transfer the embryos group by group in the corresponding glass dish (*see Note 10*) (**Fig. 3**), directly from the collection/culture medium to the fixative solution, with no rinse step (*see Note 11*).
5. Cover the glass dishes with the glass lids; in the case of a long fixation wrap each dish with parafilm to avoid drying out of the fixative at 4°C.
6. Incubate in PFA, either 20 minutes at room temperature (or 27°C on the heating plate) or overnight at +4°C.

3-2 Immunostaining

All steps are performed at room temperature (22-25°C or 27°C on the heating plate) with a total volume of 500µL per glass dish, unless otherwise mentioned. Between each step diligently follow hazardous waste recycling regulations. Carefully wash each glass dish with distilled water and dry it with a non-fluffy rag.

1. Transfer the embryos with the glass embryo manipulation pipettes from the fixative into PBS:
 - a) if PFA incubation was ≤ 30 minutes, 2 times 5 minutes PBS rinse is enough
 - b) if PFA incubation was longer, wash for at least 30 minutes (3 times 10 minutes).In both cases, and at each further step, embryos have to be aspirated in the pipette with a minimum amount of solution, breathed out in the next solution and moved within the glass dish several times (*see Note 10*) (**Fig. 3**).
2. For permeabilization, transfer the embryos and incubate 30 minutes in 0.5% TritonX100, freshly diluted in PBS (mix gently to avoid bubbles) (*see Note 12*).
3. Saturation of non-specific sites is performed by 1 hour incubation of the embryos in PBS containing 2% BSA (PBS-BSA 2%) (*see Note 13*).
4. Dilute the primary antibody with PBS containing 2% BSA in an micro test tubes tube (*see Note 14*). You will need a total of 20µl per group + 20µl for equilibration.
5. In a Petri dish prepare 20µl drops of the primary antibody for each group and add one for equilibration (*see Note 15*). Cover the drops with mineral oil (**Fig. 4**).
6. Embryos are transferred group by group in the primary antibody: first in the equilibration drop and then in their respective antibody incubation drops.
7. Incubation in the first antibody can be done either 1 hour at 37°C or overnight at 4°C (*see Note 16*). If you are using fluorescent primary antibodies, directly go to step 10 after this incubation.
8. Transfer the embryos in glass dishes of PBS. Wash 3 times 10 minutes to remove antibody excess (*see Note 17*).

9. Second antibody incubation: dilute in PBS-BSA 2% (*see Note 18*), prepare 20µl drops under mineral oil in a Petri dish (*see Note 15*) and transfer the embryos group by group as above (first in the equilibration drop and then in their respective antibody incubation drops), incubate 1 hour at room temperature (22-25°C or 27°C on the heating plate).
From this step, to the end, always carefully protect dishes from light, with aluminum foil for example.
10. Transfer the embryos in glass dishes of PBS. Wash 3 times 10 minutes to remove antibody excess (*see Note 17*).
11. Embryos can be transferred in 2% PFA (diluted in PBS) for 30 minutes + washed once with PBS (2 minutes) (*see Note 19*).
12. Transfer the embryos in glass dishes containing a nucleic acid stain diluted in PBS and incubate for 20 minutes at 37°C (*see Note 20*).

3-3 Mounting the embryos on slides

1. Prepare SuperFrost Plus slides with identification of the experiment on the side (date/species/embryonic stage/number of embryos and antibody used) and as many areas as groups drawn on the back on the slide with a permanent pen (*see Note 21 and Fig.5 A*).
2. Load a 100µl pipetman with 40µL of the antifading agent (*see Fig.5 B*).
3. Aspirate the DNA staining solution to halfway up the glass manipulation pipette and then aspirate 5 embryos of the first group, in a minimum volume of solution (*see Note 22 and Fig.5 D step 1*).
4. Breath out all the embryos, trying to put them in line in the delimited area of the slide (*see Note 22 and Fig.5 D step 2*).
5. Repeat the last two steps as many times as necessary, taking 5 embryos from the first group each time
6. In one hand, with a very thin glass pipette aspirate as much solution around the embryos as possible. In the other hand, be ready, with the pipetman containing the antifading agent to put down a little drop of antifading agent on embryos immediately after the removal of the DNA staining solution (*see Note 23 and Fig.5 D step 3*).
7. Repeat all the steps for the other groups of embryos.
8. Put a little drop of clear nail polish on each corner of the slide, next to the groups, in order to preserve the 3D-shape of the embryos (*see Fig.5 D step 4*).
9. Carefully put the cover slip on the slide helping yourself with a micro-spatula, starting from one side and getting down the other side slowly (*see Fig.5 D step 5*).
10. Secure with clear nail polish around the coverslip (*see Note 24 and Fig.5 D step 6*).
11. Keep the slides at 4°C until observation.

3-4 Microscope observation

A wide-field microscope is not adequate to capture thick specimens like three-dimensionally 3D-preserved embryos, as it does not have enough z-axis resolution. Thus 3D-preserved embryos should be observed either on a fluorescent microscope equipped with structured illumination or on a confocal laser scanning microscope. It is also necessary to use a high magnification objective adapted to fluorescent observations (X63 Plan-Neofluar for example).

1. Place the slide on the microscope with the cover slip on the same side as the objective.
2. Locate the embryos with a low magnification objective thanks to the drawings performed on the back of the slide.
3. Use high-magnification objectives to observe the immunostaining. Oil-immersion ones are usually better and require a small drop of immersion oil that should be place between the cover slip and the objective.

4. Choose the fluorescence wavelength and the emission/excitation filters corresponding to the antibodies used during the immunostaining procedure (*see Note 25*).
5. Adjust the parameters of the microscope (acquisition time for example) in order to obtain the best signal-to-noise ratio: saturation of the detector should be avoided and the background noise level should be kept to a minimum.
6. Perform Z-series acquisition with small z-steps in order to obtain the best 3D reconstruction afterwards (*see Note 26*). We also recommend taking 2-3 additional optical z-sections on top and below the limits of the staining (**Fig. 6**).
7. For comparison between groups and quantification purposes, the same objective, offset, amplifier gain, wavelength range, and dichromatic mirrors should be used for all embryos.

4. Notes

1. Ideally room temperature should be stable, between 22 to 25°C, otherwise we recommend using a heating block set at 27°C.
2. Aspirator tubes can be hand-made with HPLC clear tubing (diameter 2 millimeters) but are also commercially available (e.g. from Sigma-Aldrich). To this system you can add a filter tip and aspirate with mouth, or add a 1 or 2 ml syringe and control the valve with your hand.
3. To get embryo glass manipulation pipettes, stretch a glass capillary (or tip of a Pasteur pipette) in the flame in order to get very thin pipettes with diameters similar to the embryo ones. It is also easier to work with quite short pipettes (no more than 8 centimeters in total) and a little oblique.
4. Immunostaining is usually performed in commercially available glass dishes with one or several wells. Plastic 4-cell plates can also be used but are usually less convenient for small preimplantation embryos. The capacity of each well has to be $\geq 500\mu\text{l}$.
5. Primary antibodies are recognizing specific antigens and can be isolated from various species (mouse, rabbit, rat, human). Some primary antibodies can already be chemically linked to a fluorophore (direct fluorescent antibody). This decreases the number of steps in the staining procedure making the process faster and can reduce background signal by avoiding some issues with antibody cross-reactivity or non-specificity. However, since the number of fluorescent molecules that can be bound to the primary antibody is limited, direct immunofluorescence is less sensitive. If the fluorescent signal is too weak, amplification of the signal may be necessary. Secondary components such as fluorescently-tagged secondary antibodies (indirect immuno-staining) can then be used: multiple secondary antibodies will bind a single primary antibody and provide a more pronounced signal. This also allows more flexibility because different secondary antibodies can be used for a given primary antibody.
6. In mouse preimplantation embryos we usually start with overnight incubation at 4°C but some antibodies may be incubated 1h at 37°C in an oven or a dry incubator.
7. An extensive assortment of nucleic acid stains is commercial available for DNA counterstaining of the embryos. Their fluorescence absorption and emission spectra span the visible-light spectrum from blue to near-infrared, making them compatible with many different types of secondary antibodies. We often use DAPI (blue), Yo-Pro-1 (green), Propidium Iodide (red) and Ethidium Homodimer 2 (red).
8. Glass cover slips are commercially available in a variety of widths, lengths, and thicknesses. We recommend square or rectangular ones, easier to place on the embryos,

measuring 22*22mm up to 22*40mm. The thickness of the cover slip is crucially important for high-resolution microscopy. Most microscope objectives are designed for use with No. 1 cover slips (0.13 to 0.17 mm thickness). Use of cover slips that deviate from this thickness will result in spherical aberration and a reduction in resolution and image intensity.

9. A comparative experiment with a short fixation and a long one in parallel, using the same antibodies, should of course be performed to confirm that a long fixation step does not alter the immunostaining.
10. As embryos are known to be very sensitive to culture conditions and environmental changes, we advise to perform embryo fixation as quickly as possible and to transfer the directly from the collection/culture medium to the fixative solution, with no rinse step.
11. The global success of the immunostaining, especially the quality of the signal, resides in the perfect contact of the embryos with each incubation solution. It is thus necessary at each step to properly transfer the embryos and move them in the glass dish as described (**Fig. 3**).
12. In some cases, when the immunodetection is not giving a good signal, permeabilization may be either 1) extended to 1 hour, 2) performed with 1% Triton X-100, 3) performed at higher temperature, e.g. 37°C or 4) after removal of the zona pellucida by HCl or pronase treatments. This last step is very useful in other species than mouse.
13. If necessary you can add, after the saturation of non-specific sites, a step of denaturation with HCl (2N HCl at 37°C for 1 hour) in order to increase DNA accessibility to the primary antibodies. This is particularly important for antibodies directed against 5-MeC or 5-hMeC (**14**).
14. Antibodies have to be prepared in sterile conditions, either under an appropriate hood or next to a flame. The dilution of the primary antibody will be adjusted in preliminary experiments. The best is to determine the lowest concentration that still provides an immunostaining signal, with a good signal-to-noise ratio. You may also combine several primary antibodies if they are not derived from the same species (to avoid cross-reactivity upon addition of the secondary antibodies) or if they are direct fluorescent antibodies. Combining several primary antibodies however requires preliminary experiments with each individual antibody to control that they do not physically cross-react with each other. In order for example to combine 5-MeC or 5-hMeC immunodetection with the detection of modified histones we recommend to proceed in several steps: 1) incubate with the primary antibody directed against the modified histone(s), 2) wash with 0.05% Tween-20 in PBS for 30 minutes, 3) post-fix in 4% PFA for 25 minutes, 4) treated with HCl as recommended in **Note 13** and 5) incubate with the 5-MeC or 5-hMeC primary antibody.
15. If the immunostaining results are not satisfactory, showing too much background, especially big fluorescent dots due to antibodies aggregates, we recommend centrifuging the antibody (10 minutes at 13 000 rpm with a microcentrifuge) after the dilution, before taking the supernatant to prepare the drops.
16. Choice of the incubation length and temperature will be adjusted in preliminary experiments in order to find the conditions that provide the best signal-to-noise ratio.
17. 0.05% of Tween 20 may be added in these washing steps in order to reduce the background signal if necessary.
18. If you want to work on several proteins and use several antibodies simultaneously, make sure that 1) primary antibodies are produced in different species, 2) each corresponding secondary antibody is coupled to a different fluorophore and 3) that each chosen fluorophore can be distinguished by the microscope that will be used. Secondary antibody dilution will be selected according to the manufacturer's recommendations.

We commonly use 1/200 or 1/300 dilutions with antibodies from Jackson ImmunoResearch.

19. The “post-fixation” is used to preserve the immunostaining when immediate observation on the microscope is not possible.
20. DNA stains concentrations: 1µg/ml DAPI; 0.002 mM Ethidium Homodimer-2; 1µg/ml Propidium Iodide.
21. Do not use the extremity of the slide: when using an inverted microscope for observations the objective could be damaged.
22. Each step has to be done carefully in order to preserve the 3D-shape of the samples.
23. Be particularly careful to not dry the embryos.
24. Do not pushing on the coverslip in order to avoid squashing.
25. Fluorescence wavelength and emission/excitation filters can be found on the antibodies manufacturers’ websites. We recommend the Fluorescence SpectraViewer from Invitrogen, Life Technologies that allows all kinds of combinations and comparisons.
26. The minimal z-step will depend on the resolution in the depth direction of your microscope that is determined by the numerical aperture of the objective, the refractive index of the objective immersion media and the wavelength of the light used.

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FIGURES

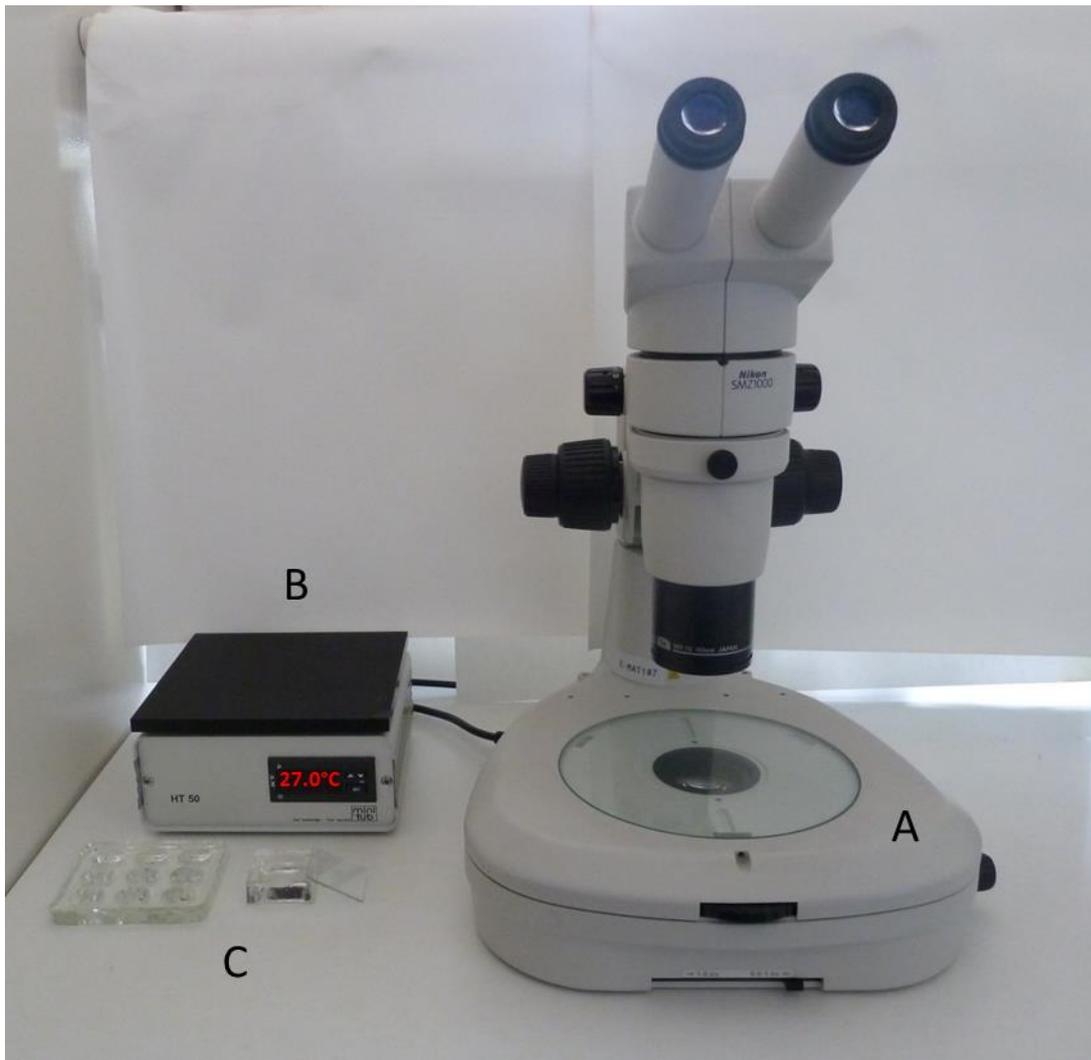


Figure 1: Basic equipment required.

Performing immuno-staining on whole-mount preimplantation embryos requires A) a dissecting microscope with appropriate working distance, B) an heating block set at 27°C and C) embryo glass dishes (with one or several wells) with glass lids.

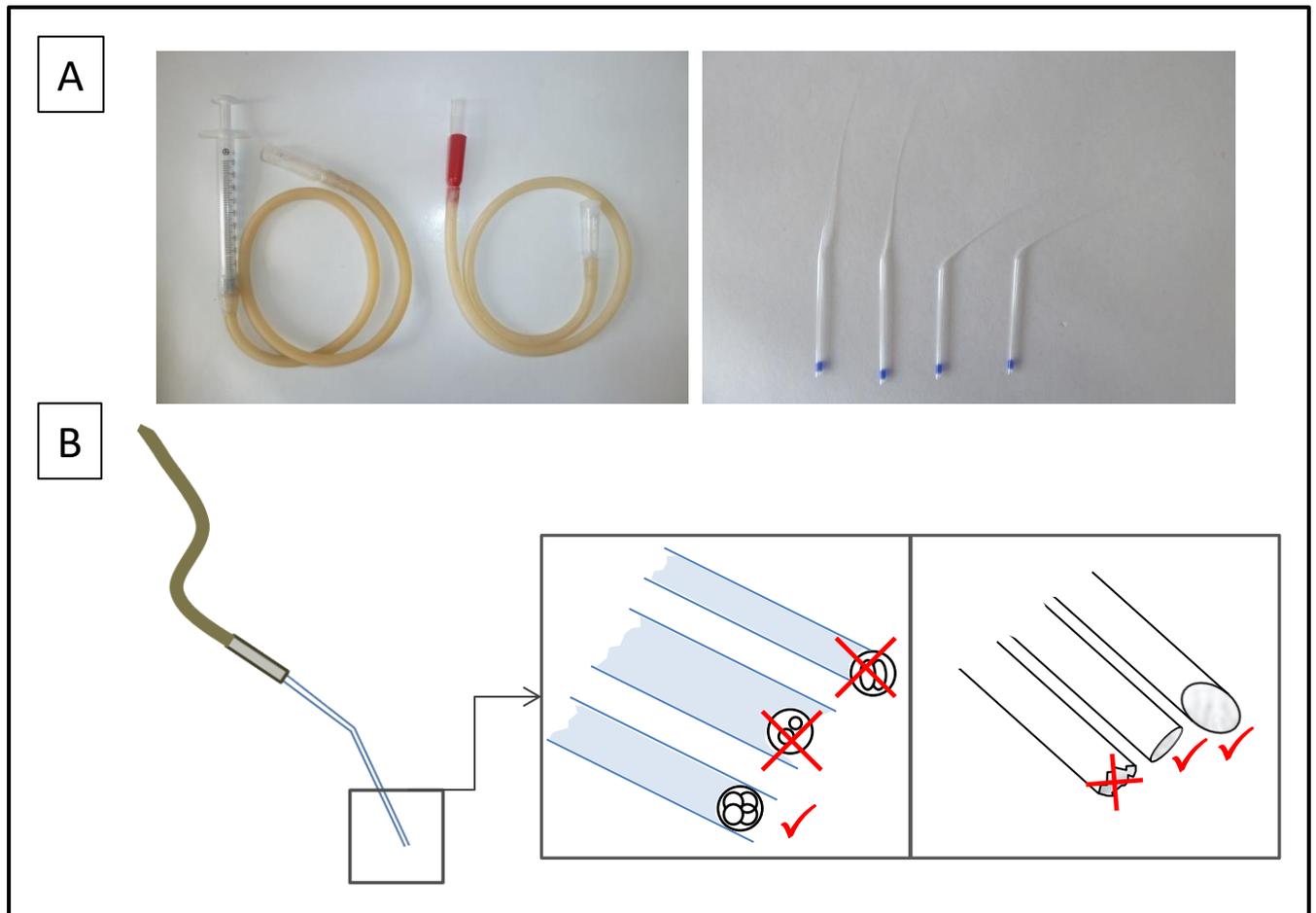


Figure 2: Aspirator tube and glass manipulation pipettes.

- A) Examples of aspirator tubes with either syringe or mouth control and of some glass manipulation pipettes (straight and oblique)
- B) Choice of the right diameter for the manipulation of the embryos: it should fit exactly to the diameter of the embryos and have smooth extremity. If necessary the diameter can be increased by breaking carefully (with your nails or with a diamond pen) the extremity of the glass pipette.

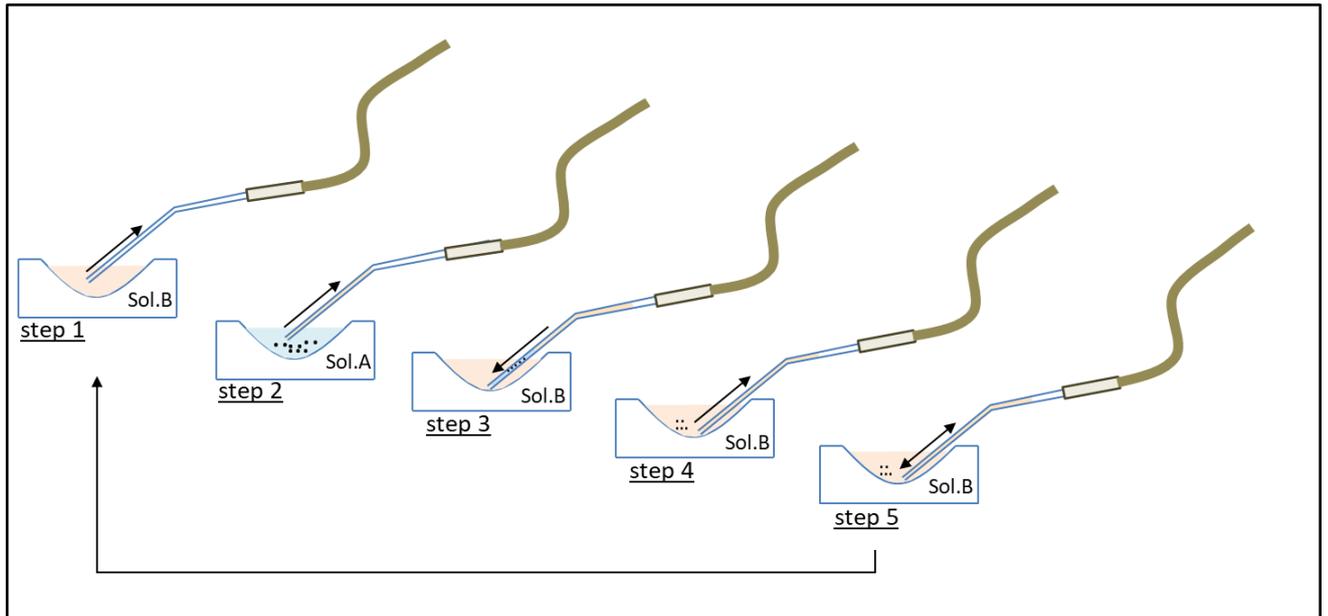


Figure 3: Embryo transfer step by step.

The quality of the immunostaining resides in the perfect contact of the embryos with each incubation solution. For each transfer from solution A to solution B, follow the steps as described:

- Step 1: aspirate solution B to halfway up the glass manipulation pipette
- Step 2: aspirate 5 embryos, in a minimum volume of the solution A
- Step 3: breathe out the embryos in solution B and discard the rest of solution A
- Step 4: aspirate solution B to halfway up the glass manipulation pipette
- Step 5: move the embryos around in the glass dish several time

Repeat the 5 steps as many times as necessary, taking 5 embryos each time

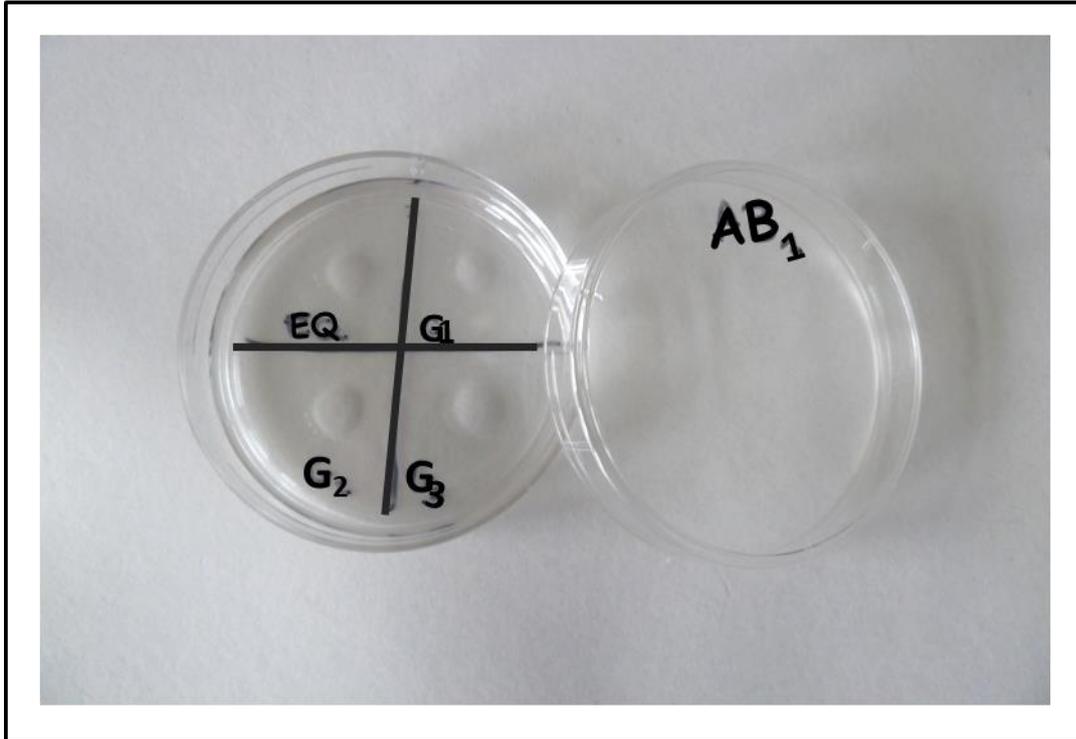


Figure 4: Antibody incubation.

For primary and secondary antibody incubations prepare for each group (G1, G2, G3 etc...) 20µl drops of the antibody (diluted with PBS containing 2% BSA) and add one drop for equilibration (EQ). Cover the drops with mineral oil and transfer the embryos group by group: first in the equilibration drop and then in their respective antibody incubation drops.

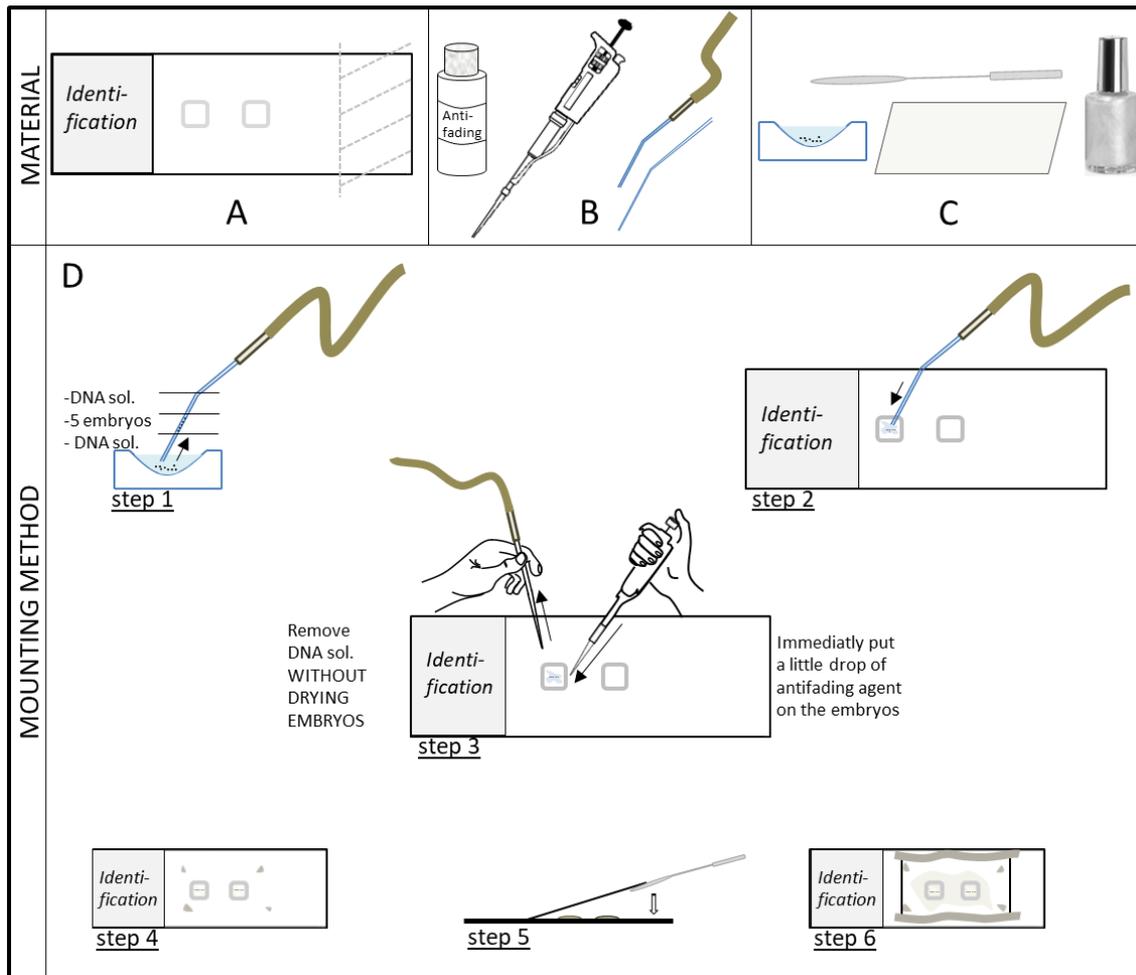


Figure 5: Mounting of the immunostained embryos on slides.

Mounting the embryos on slides has to be done carefully in order to preserve the 3D-shape of the samples. First prepare the following material: A) SuperFrost Plus slides with identification of the experiment on the side (date/species/embryonic stage/number of embryos and antibody used) and as many areas as groups drawn on the back on the slide with a permanent pen (do not use the extremity of the slide : when using an inverted microscope for observations the objective could be damaged); B) 100µl pipetman ready to use with 40µL of the antifading agent and two glass manipulation pipettes (a perfectly calibrated and a very thin one with a diameter smaller than the embryos); C) the immunostained embryos in their glass dishes, a glass cover slip, a micro-spatula and clear nail polish to secure the cover slip into place. Then follow the mounting steps:

- Step 1: aspirate the DNA staining solution to halfway up the glass manipulation pipette and then aspirate 5 embryos of the first group, in a minimum volume of the DNA staining solution.
- Step 2: breathe out all the embryos, trying to put them in line in the delimited area on the slide.
- Repeat steps 1 and 2 as many times as necessary, taking 5 embryos from the first group each time.
- Step 3: in one hand, with a very thin pipette aspirate as much solution around the embryos as possible, however be particularly careful to not dry the embryos / in the other hand, be ready, with the pipetman containing the antifading agent to put down a little drop of antifading agent on embryos immediately after the removal of the DNA staining solution.
- Repeat all the steps for the other groups of embryos.
- Step 4: put a little drop of clear nail polish on each corner of the slide, next to the groups, in order to preserve the 3D-shape of the embryos.
- Step 5: carefully put the cover slip on the slide helping yourself with a micro-spatula, starting from one side and getting down the other side slowly.
- Step 6: secure with clear nail polish around the coverslip, without pushing on it in order to avoid squashing of the embryos.

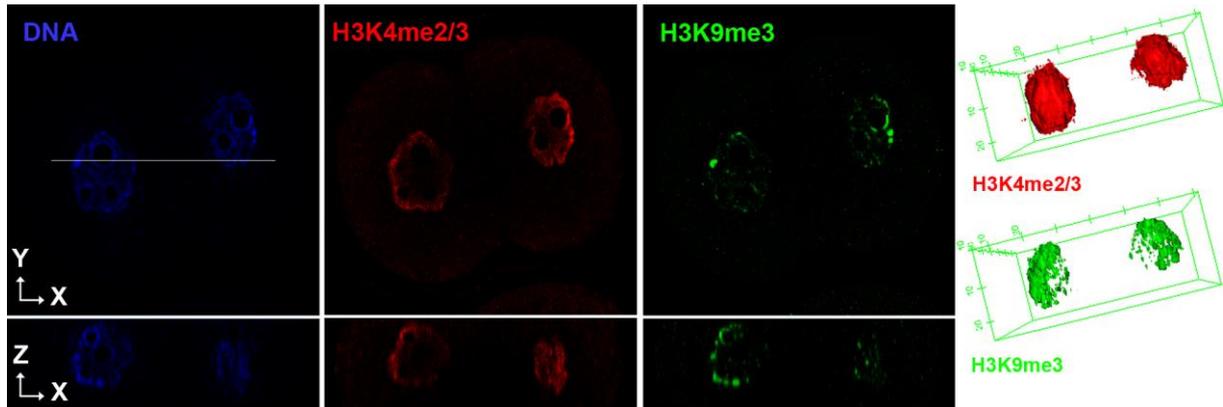


Figure 6 : Immunodetection of two epigenetic modifications in a 2-cell mouse embryo produced by nuclear transfer.

Two different epigenetic modifications (H3K4me2/3 in red and H3K9me3 in green) are immuno-stained. DNA is counterstained in blue. The figure presents confocal sections according to the classical X/Y axis but also according to the X/Z axis showing the depth of the nuclei, at the position indicated by the white line. The 3D- shape of the nuclei is clearly preserved. The corresponding 3D reconstructions obtained with the 3D Viewer Plugin from ImageJ are shown on the right. It shows that whereas H3K4me2/3 staining is present in the whole nuclei, H3K9me3 aggregates preferentially on one side of each nucleus.