Video Article

Analysis of Pluripotent Stem Cells by using Cryosections of Embryoid Bodies

Ismael C. Gomes*[1], Mariana Acquarone*[1], Renata de Moraes Maciel*[1], Rafael Bierig Erlich[1], Stevens K. Rehen[1]

[1]Laboratório Nacional de Céulas-Tronco Embrionárias (LaNCE), Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro (UFRJ), Brazil

*These authors contributed equally

Correspondence to: Stevens K. Rehen at srehen@anato.ufrj.br

URL: https://www.jove.com/video/2344
DOI: doi:10.3791/2344

Keywords: Developmental Biology, Issue 46, Embryonic stem cells, embryoid body, cryosections, immunochytochemistry, H9

Date Published: 12/8/2010

Citation: Gomes, I.C., Acquarone, M., Maciel, R.d., Erlich, R.B., Rehen, S.K. Analysis of Pluripotent Stem Cells by using Cryosections of Embryoid Bodies. *J. Vis. Exp.* (46), e2344, doi:10.3791/2344 (2010).

Abstract

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst-stage early mammalian embryos [1]. A crucial stage in the differentiation of ES cells is the formation of embryoid bodies (EBs) aggregates [2, 3]. EB formation is based on spontaneous aggregation when ES cells are cultured in non adherent plates. Three-dimensional EB recapitulates many aspects of early mammalian embryogenesis and differentiate into the three germ layers: ectoderm, mesoderm and endoderm [4].

Immunofluorescence and *in situ* hybridization are widely used techniques for the detection of target proteins and mRNA present in cells of a tissue section [5, 6, 7]. Here we present a simple technique to generate high quality cryosections of embryoid bodies. This approach relies on the spatial orientation of EB embedding in OCT followed by the cryosection technique. The resulting sections can be subjected to a wide variety of analytical procedures in order to characterize populations of cells containing certain proteins, RNA or DNA. In this sense, the preparation of EB cryosections (10µm) are essential tools for histology staining analysis (e.g. Hematoxilin and Eosin, DAPI), immunofluorescence (e.g. Oct4, nestin) or *in situ* hybridization. This technique can also help to understand aspects of embryogenesis with regards to the maintenance of the tri-dimensional spherical structure of EBs.

Video Link

The video component of this article can be found at https://www.jove.com/video/2344/

Protocol

1. Fixation and Cryopreservation

Pluripotent stem cells were cultured onto mouse embryonic fibroblasts (MEF) inactivated with mitomycin C and maintained in DMEM/F12 supplemented with 20% knockout serum replacement (KSR) and 8ng/ mL of fibroblast growth factor (FGF-2). In order to induce EB formation H9 cells were transferred to non-adherent dishes and cultured for 7 days, maintained in DMEM/F12 supplemented with 15% KSR [8].
Note (!): This technique can be used for EBs derived from any embryonic and induced pluripotent stem cell.

1. Collect the EBs from the culture dish with a pipette.
2. Transfer EBs to a 15 mL conical tube and wait until the material sinks to the bottom of the tube.
3. Remove medium and fix EBs with a 4% paraformaldehyde (PFA) solution in PBS for 30 minutes in room temperature.
   Note (!): For *in situ* hybridization antigen recovery should be done in order to avoid cross link reaction with antibodies due to the use of PFA[6,7].
4. Remove PFA solution and wash with PBS for 5 min.
5. EBs should be placed in serial dilution of PBS-buffered sucrose solutions (10, 20 and 30%, in sequence) at 25-28°C, each solution must be replaced every 30 min. The material can then be stocked in the 30% sucrose solution at 4°C until the embedding step with OCT.

2. Tissue Embedding, Guidance and Freezing

6. Carefully collect the EBs from the tube. Discharge as much sucrose solution as possible.
   Note (!): It is important to wet the internal wall of the tip with sucrose solution before collecting the EBs. This procedure can avoid EB to be attached to the tip.
7. Place EBs in the mold and remove remaining sucrose solution with filter paper.
   Note (!): It is important not to fill the mold with EBs to avoid overlapping.
8. Fill mold with OCT slowly, taking care not to re-suspend EBs. After that, place all EB at the center of the mold and remove bubbles with the pipette.
9. Mildly agitate for 15 minutes.

10. Surround the mold with crushed dry ice to freeze the sample. OCT should be completely frozen. At this point blocks can be stored at -70°C, for at least one year.

3. Cryosectioning Technique

11. Remove the frozen block from -70°C and place on cryostat previously cooled at a temperature between -18 and -21 °C.
    Note (!): Temperatures outside this range may cause troubles like sections curling, melting or cracking.
12. Detach the OCT block from the mold and place it into the cryostat support by adding more OCT.
13. It is important to orient the block properly and align it parallel to the edge of the blade.
    Note(!): As EBs are smaller than other tissue samples, this step is extremely important to minimize loss of material.
14. OCT blocks should be sectioned superficially until the surface plane is obtained. Thin sections (10µm) of the tissue specimen must be taken and mounted onto glass slides previously coated with 200 mg/mL poly L lysine (10 µL per slide) [9,10].
    Note(!): To avoid torned sections pay attention to any damages at the blade.
15. All sections should be air-dried for 1 hour at room temperature and then be used or stored at -70°C until use.

Discussion

The method described here provides an easy-to-follow protocol to obtain PFA fixed thin cryostat sections of embryoid bodies useful for immunofluorescence and *in situ* hybridization assays. The resulting cryosections permit the study of cellular and molecular aspects of human embryonic stem cells differentiation, while preserving their structure and organization as aggregates.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Instituto Nacional de Ciência e Tecnologia (INCTC). We are grateful to Bruna S. Paulsen and Aline M. Fernandes for the EB images.

References

1. Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., & Jones, J.M. Embryonic stem cell lines derived from human blastocysts. *Science.* 282, 1145-7 (1998).
2. Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., & Benvenisty, N. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med.* 6. 88-95 (2000).
3. Kurosawa, H. Methods for inducing embryoid body formation: *in vitro* differentiation system of embryonic stem cells. *J Biosci Bioeng.* 103. 389-398 (2007).
4. Conley, B.J., Young, J.C., Trounson, A.O., & Mollard, R. Derivation, propagation and differentiation of human embryonic stem cells. *Int J Biochem Cell Biol.* 36. 555-567 (2004).
5. Moon, I.S., Cho, S.J., Jin, I., Walikonis, R. A simple method for combined fluorescence *in situ* hybridization and immunocytochemistry. *Mol Cells.* 24(1):76-82 (2007).
6. Daneshtalab, N., Doré JJ, Smeda JS. Troubleshooting tissue specificity and antibody selection: Procedures in immunohistochemical studies. *J Pharmacol Toxicol Methods.* 61(2):127-35 (2009).
7. Krenacs, L., Krenacs T, Stelkovics E, Raffeld M. Heat-induced antigen retrieval for immunohistochemical reactions in routinely processed paraffin sections. *Methods Mol Biol.* 588:103-19 (2010).
8. Nones, J., Spohr TC, Furtado DR, Sartore RC, Paulsen BS, Guimarães MZ, Rehen SK. Cannabinoids modulate cell survival in embryoid bodies. *Cell Biol Int.* 12. 399-408 (2010).
9. Huang, W.M., Gibson SJ, Facer P, Gu J, Polak JM. Improved section adhesion for immunocytochemistry using high molecular weight polymers of $_L$-lysine as a slide coating. *Histochemistry.* 77(2):275-9 (1983).
10. Kuenzi, M.J. and Sherwood OD. Immunohistochemical localization of specific relaxin-binding cells in thecervix, mammary glands, and nipples of pregnant rats. *Endocrinology.* 136: 1367-1373 (1995).

