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Short communication

A reliable strategy for single-cell RNA sequencing analysis using cryoconserved primary cortical cells

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ABSTRACT

Background: The application of single-cell RNA sequencing (scRNASeq) represents a unique approach to identify hundreds to millions of cells in mammalian cortical multilayers at different stages of embryogenesis. ScRNASeq technology applied to neurological studies requires the use of fresh starting materials because standard cryopreservation methods do not guarantee high viability of cortical primary cells derived from dissected brain areas.
New method: Here we set up and validate an innovative strategy to perform scRNASeq studies in cryopreserved primary cortical cells isolated from E15.5 mouse embryo. In order to freeze cortical primary cells, we have employed Neurostore, a medium able to guarantee high viability and cell composition of embryonic cortex after thawing.
Comparison with existing methods: We showed for the first time the possibility to run scRNASeq experiments on primary cortical cells in an off-line set-up, ensuring cellular integrity and diversity.
Results: By trypan blue assay and flow cytometry analysis, we found that Neurostore-cryopreserved cortical cells showed approximately 95 % of viability. Satisfactory RNA recovery and cDNA libraries were achieved. Transcriptome sequencing of 35,763 cryoconserved single cells yielded a robust data-set, identifying 25 cell clusters in three biological samples. Prevalence of peculiar neural populations before and after the cryopreservation-resuscitation procedure was verified by marker gene expression and immunofluorescence analysis.
Conclusions: Our findings support the evidence that frozen primary cortical cells can be successfully employed in scRNASeq experiments allowing an unprecedented flexibility in experimental procedures, such as sample preparation and subsequent processing steps performed in different locations.

1. Introduction

Single-cell RNA sequencing (scRNASeq) is a break-through technology that measures gene expression at the resolution of individual cells. It presents exciting opportunities to study gene expression heterogeneity and to characterize the overall cell types. The complexity of mammalian brain composed by thousands of different cell types and subtypes with unique morphology and gene expression profiles can be explored by scRNASeq technology (Loo et al., 2019; Zeisel et al., 2018, 2015). Unlike bulk transcriptomic analysis, single-cell transcriptional profiling of the embryonic nervous system provides a clear picture of cells diversity and the possibility to evaluate the interaction between neuronal and non-neuronal cell types (Chen et al., 2019). To reach this goal, cell integrity and viability as well as RNA quality are crucial technical requirements. Storage of tissues and cells allows archiving of biological samples, replication of experiments from the same source and facilitates

Abbreviations: scRNASeq, single-cell RNA sequencing; FCM, flow cytometry analysis; GEMs, gel bead-in-emulsions; UMI, single-cell gene unique molecular identifier; UMAP, uniform manifold approximation and projection.

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collaboration among different laboratories based at distant locations. However, standard cryoconservation methods lead to crystallization and disruption of biological structures, such as cellular membranes, which prevents subsequent cell preparation for high-throughput technologies. In the last years, many different methods have been developed for testing cryopreservation of primary neuronal cultures in order to preserve their morphology and viability in rodents (Parker et al., 2018; Pischedda et al., 2018; Ishizuka and Bramham, 2020). In detail, Pischedda et al. (2018) developed a new medium called Neurostore, which allowed not only to preserve murine cell viability but also to protect typical neuronal features, including neurite arborization, synaptic density and electrophysiological activity.

Here, we describe a new protocol to gain single-cell transcriptome sequencing on 10X Genomics Chromium instrument of cryopreserved primary cortical cells prepared from E15.5 male mice.

To cope with potential biases due to cell sampling, differences in the mRNA molecule number, library size and clustering, we carried out a global bioinformatic normalization prior to analyzing biological signals (Hicks et al., 2018; Yuan et al., 2017).

2. Materials and methods

2.1. Animals

All experiments in mice were conducted in conformity with the European Community Directive 2010/63/EU and were approved by the Italian Ministry of Health (DLgs116/92) in accordance with the Institutional Animal Care guidelines of the Institute of Genetics and Biophysics "Adriano Buzzati-Traverso", under the accreditation n°307/2018-PR E58D.8.

C57BL/SJ wild-type (WT) mice, purchased from Charles River Laboratories, were kept under standard conditions with water and food supplied *ad libitum*. WT pregnant animals at embryonic day 15.5 (E15.5) of gestation were anesthetized with diethyl ether and sacrificed by cervical dislocation. Embryonic age was calculated by day 0.5 of pregnancy when the vaginal plug was detected. Mouse embryos at E15.5 were carefully isolated from the uterus and then genomic DNA from tail tissue of each embryo was extracted. Sex determination of male embryo was performed by PCR amplification of *Sry* gene (Table S2).

2.2. Cortical dissection

E15.5 C57/BL6 WT embryos were decapitated and whole brains were isolated from skulls. Successively, meninges were carefully removed under the light microscope. Cortical hemispheres were collected upon removing the brainstem, cerebellum, and midbrain following standard methods (Han et al., 2009; Loo et al., 2019). All these procedures were done in ice-cold dissection medium 1X PBS (Mg^{2+} and Ca^{2+} free; Gibco), supplemented with 33 mM D-glucose (Bioshop) and 0.1 mg/mL penicillin/streptomycin (Gibco). Successively, dissected cortices were centrifuged for 7 min at 400 RCF and successively resuspended in Eagle's Minimum Essential Medium (MEM, Gibco) supplemented with 5% Fetal bovine serum (FBS, Gibco), 5% Horse serum (Gibco), 20 µM Gentamycin (Gibco), 500 µM L-glutamine (Gibco) and 1.5 mM D-glucose (Bioshop). Tissue samples were mechanically dissociated with a glass pipette, until cell clusters were no more present and cell suspensions were obtained (Mancini et al., 2014).

2.3. Cryopreservation and thawing of primary cortical cells

Primary cellular suspensions were centrifuged at 100 RCF for 5 min at room temperature. Cellular pellets were resuspended in 1 mL of Neurostore freezing medium (Pischedda et al., 2018) or 1 mL of pre-chilled (4 °C) 10 % dimethyl sulfoxide (DMSO) diluted in Hank's Buffered Salt Solution (HBSS; 1:9; Rahman et al., 2010; Wohnhaas et al., 2019). Aliquots of 1 mL were dispensed into cryovials and placed into a freezing container (Nalgene) that was previously filled up with isopropyl alcohol (Sigma) and finally stored at −80 °C. Frozen samples were rapidly thawed in a water bath at 37 °C in continuous agitation (2−3 min). For Neurostore-cryoconserved samples, cells were resuspended in pre-warm Thaw medium (Pischedda et al., 2018), while DMSO-cryoconserved cells were resuspended in HBSS 1 ×. Each diluted cell suspension was centrifuged for 5 min at 100 RCF, the supernatant was carefully removed and the pellet was gently resuspended in 1 mL MEM medium. Finally, primary cortical cells were counted by standard trypan blue method (Wohnhaas et al., 2019). RNA extraction, qPCR and Immunofluorescence studies were done following standard protocols as described in supplementary data section (SupData).

2.4. Cell preparation for single-cell RNASeq study

Neurostore-cryopreserved cells were seeded on Poly-D-lysine (1 mg/mL, Sigma) coated 6-well plates (Corning) and cultured in MEM at 37 °C and 5% CO2 for 16−18 h approximately. Single-cell suspensions were obtained upon incubation at 37 °C for 10 min with 0.05 % Accutase (Biowest). Successively, cells were collected and centrifuged for 10 min at 100 RCF at room temperature. Cells were resuspended in 0.04 % Bovine Serum Albumin (BSA, Sigma)/1X PBS as recommended in 10x Genomics® Single Cell protocol. Counting of single-cell suspension was assessed by trypan blue exclusion staining with bright field optics. Finally, each single-cell suspension was centrifuged for 3 min at 100 RCF and resuspended in 0.04 % BSA/1X PBS to a final concentration of 1×10^3 cells/µl.

2.5. Flow cytometry analysis

Viability of single-cell suspension was evaluated by flow cytometry (FCM) using propidium iodide [4 µg/mL] (Sigma). The analysis was performed with at least 10.000 cells per sample using a CANTO II flow cytometer equipped with Diva Software (BD Immunocytometry Systems).

2.6. Statistical analysis

The statistical analyses were performed with the GraphPad Prism 7 software. Student *t*-test was used to assess statistical comparison among the experimental groups reported as HBSS/ 10 % DMSO vs. Neurostore and Pre-plating vs. Post-plating cell counting. P-values <0.05 were considered significant.

2.7. Single-cell RNASeq experiments

Twenty thousand cultured cortical neurons from three independent WT embryos (#1, #2 and #3) were treated as recommended in 10x Genomics® Single Cell protocol (v3.1 chemistry, https://www.10xgenomics.com/). scRNASeq libraries, diluted 1:4, were run on Agilent TapeStation High Sensitivity D1000 screen tape to check the quality assessment. Finally, all samples were sequenced on Illumina HiSeq2500 instrument by a paired end run (28 cycles for read1, 91 cycles for read2) in order to obtain almost 20,000 reads per cell.

2.8. Processing of scRNASeq data and cluster identification

Data derived from scRNASeq were processed into transcript count tables with the Chromium Single Cell Software Suite by 10x Genomics®. Cell filtering, data normalization, and unsupervised clustering were carried out using the R package Seurat version 3.0.1 (http://satijalab.org/seurat/) (Butler et al., 2018; Macosko et al., 2015) (see SupData). The original Louvain graph-based clustering algorithm was used to cluster cells, using a resolution of 1.2. The three datasets were integrated using the Harmony package (version 0.99.9, https://github.com/immunogenomics/ Harmony) (Korsunsky et al., 2019), implemented in R

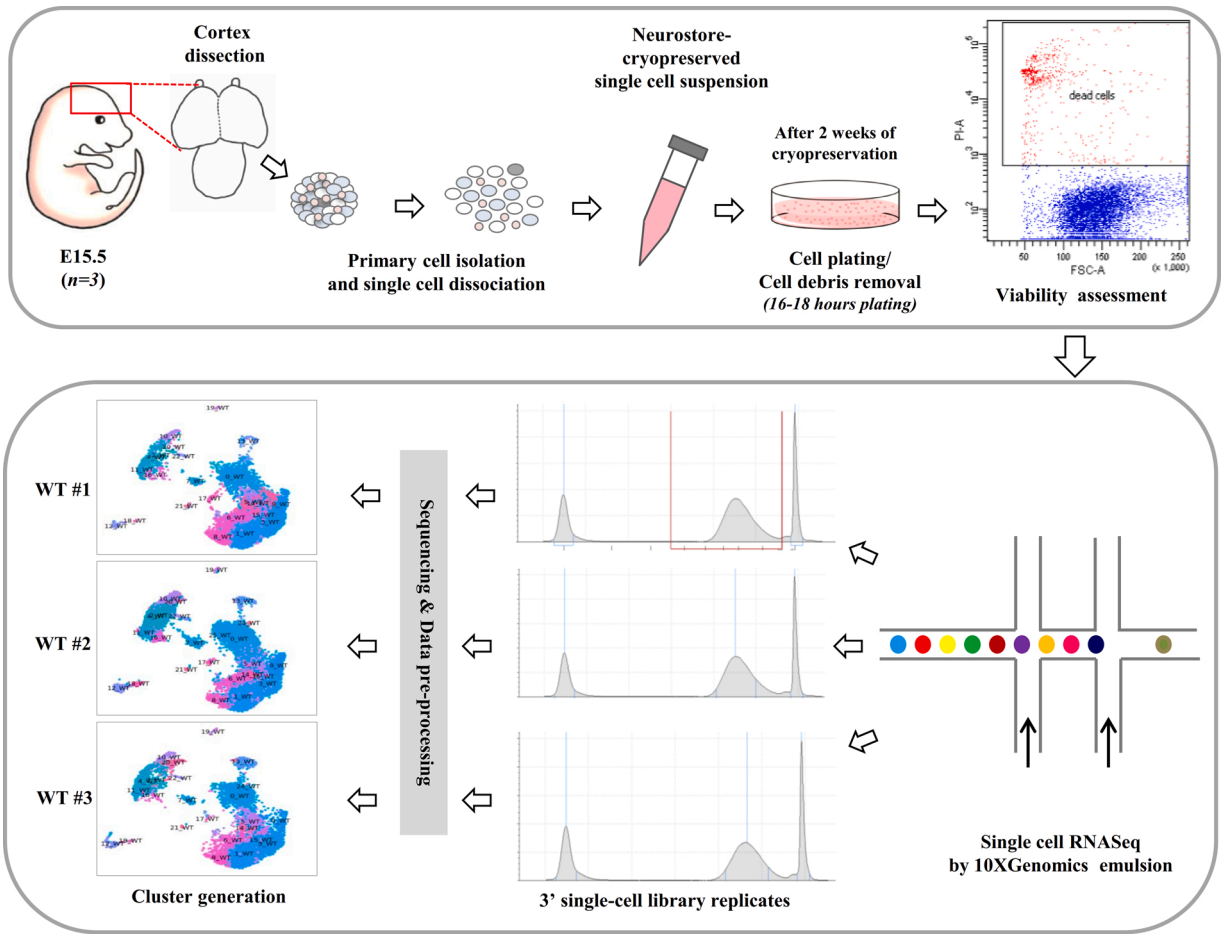

Fig. 1. Graphical abstract. Workflow of single-cell analysis in Neurostore-cryoconserved murine cortical cells. Primary cortical cells isolated from E15.5 male mouse cortex are cryopreserved in Neurostore medium and stored at −80 °C or liquid nitrogen. Upon plating, cell viability is assessed by flow cytometry. Library construction, sequencing, data pre-processing and cluster identification are performed following 10XGenomics scRNASeq protocol.

environment, in order to avoid batch effects in the downstream analysis. To evaluate the correction results, we employed Uniform Manifold Approximation and Projection (UMAP) visualizations (McInnes et al., 2018). Considering expression values of marker genes in E15.5 neocortex cell populations (Cajal-Retzius cells, layer neurons, neuron progenitors and neural stem cells), a heat map data was performed by using GraphPad.

3. Results and discussion

The achievement of efficient cryopreservation of primary murine neuronal cells is instrumental when it is necessary to disconnect time and location of sample collection from subsequent processing steps for single-cell sequencing experiments.

Here we set up and validate a new approach that by-passes the necessity of fresh starting material, leading toward new applications in neuroscience research. In fact, we were able to generate a representative map of the developing cortex at a single cell level, coupling single-cell dissociation and isolation, high-quality library construction, sequencing and bioinformatic analysis (Conesa et al., 2016). We propose here a workflow implying cryopreserved primary murine cortical cells and single-cell RNASeq technology (Fig. 1).

3.1. Evaluation of cell viability of cryopreserved primary cortical cells

A successful single-cell sequencing study depends on the quality and numerousness of the single-cell preparation used per experiment. Ideally, only live cells are fed into the system, and therefore, cell dissociation and storage of samples are essential. We prepared primary cortical cells from three WT E15.5 embryos obtained *via* a standard dissociation protocol (Mancini et al., 2014). The single-cell suspensions were cryopreserved at −80 °C in two different media, HBSS/10 % DMSO and Neurostore, aiming to guarantee high viability (>80 %) and to reproduce cell composition of embryonic cortex (Loo et al., 2019). First, we tested the presence of non-viable cells by trypan blue exclusion staining in cryoconserved samples for two weeks. In line with previous study proving that only 30 % Neurostore-cryopreserved neurons were dead cells (Pischedda and Piccoli, 2019), we found that approximately 72 % Neurostore-cryopreserved cells were viable (28 % cell death), while HBSS/10 % DMSO medium yielded only 17 % viable cells (Fig. 2A). Next, we removed all dead cells or debris from cell samples. To this end, we seeded cortical cell suspensions on Poly-D-Lysine coated-plates. After 16−18 h we removed cell media and treated cultures with Accutase to obtain again a single-cell suspension. Noteworthy, no significant difference in live cells count was observed before and after plating (Fig. 2B). In addition, FCM analysis using propidium iodide staining, indicated that approximately 95 % post-plating cryopreserved cells were viable (Fig. 2C), whose roughly 86 % were single cells (Fig. 2C). Furthermore, we analyzed cell viability after three- and five-weeks storage at -80 °C establishing that 90 % of cells remained viable in both conditions (Fig. S1). Our results strongly support the applicability of cryopreserved cultures in scRNASeq experiments: cryopreservation allows extreme flexibility in setting up scRNASeq experiments.

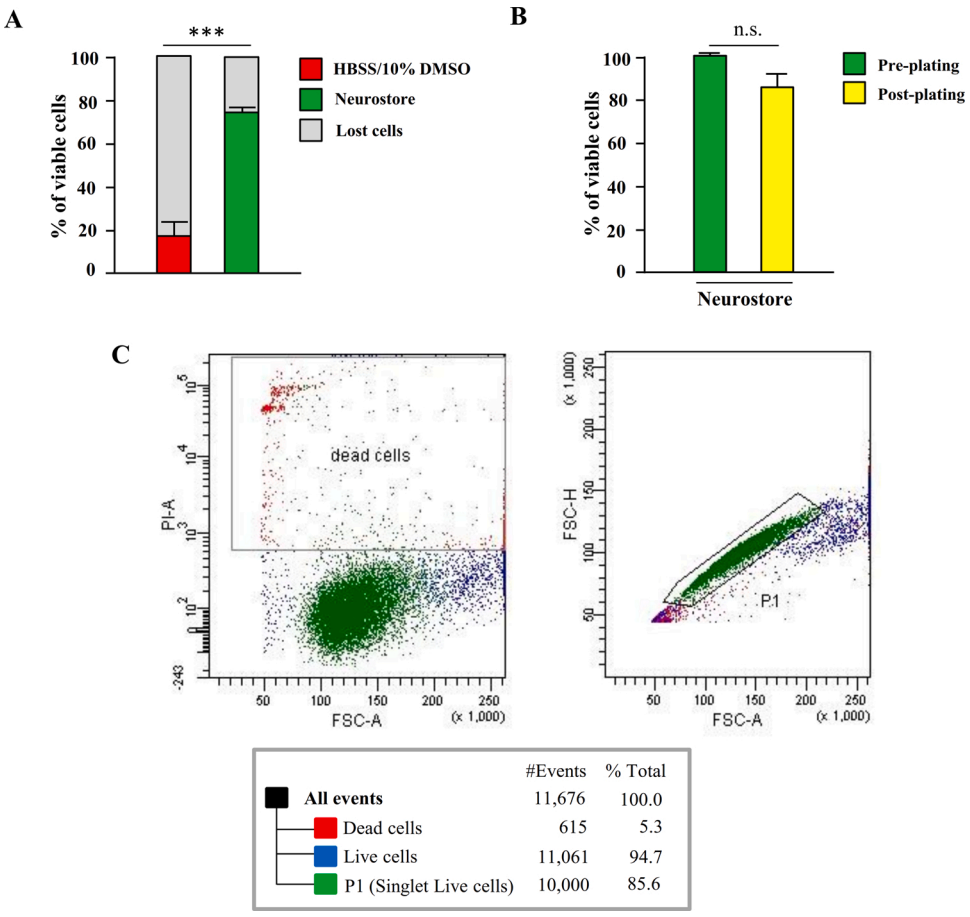

Fig. 2. Cell counting and viability analysis of cryopreserved cortical cells. Evaluation by Trypan blue exclusion assay of viable and dead cells upon cryogenic storage in HBSS/10 % DMSO or Neurostore medium (A) and of Neurostore cryopreserved viable cells before (pre-plating) and after plating (post-plating) (B). Data are reported as mean ± standard deviation of three independent experiments (*** =p < 0.0005). n.s. = not significant. (C) FCM analysis in post-plating Neurostore-cryopreserved cells. *On the left panel*, cells were gated based on FSC-A (Forward Scatter Area) and fluorescence intensity for Propidium Iodide (PI). Live cells (*below* the black line) and dead cells (*above* the black line) populations are shown. *On the right panel*, cells were gate based on FSC-A and FSC-H (Forward Scatter Height) to gate out singlet cells (P1 population).

3.2. *Single-cell experiment: pre-processing data*

To evaluate the performances of the Neurostore-cryoconserved primary cortical cells, we employed unbiased high-throughput scRNASeq of twenty thousand cortical cells, isolated and counted from each single-cell suspension (WT E15.5 #1, #2 and #3). To minimize possible batch effects, all cryopreserved primary cortical cell suspensions of the three biological replicates were loaded on different well positions on the same chip and the libraries were run on separate Illumina sequencing lanes. We transcriptionally profiled 35,763 estimated cells from the three independent groups. In detail, we recovered independently 10,419 cells from WT embryo #1, 18,571 from WT embryo #2 and 6773 from WT embryo #3 (Table S1). After amplifying the cDNA, the molecules were randomly fragmented and, as expected, the prepared libraries showed

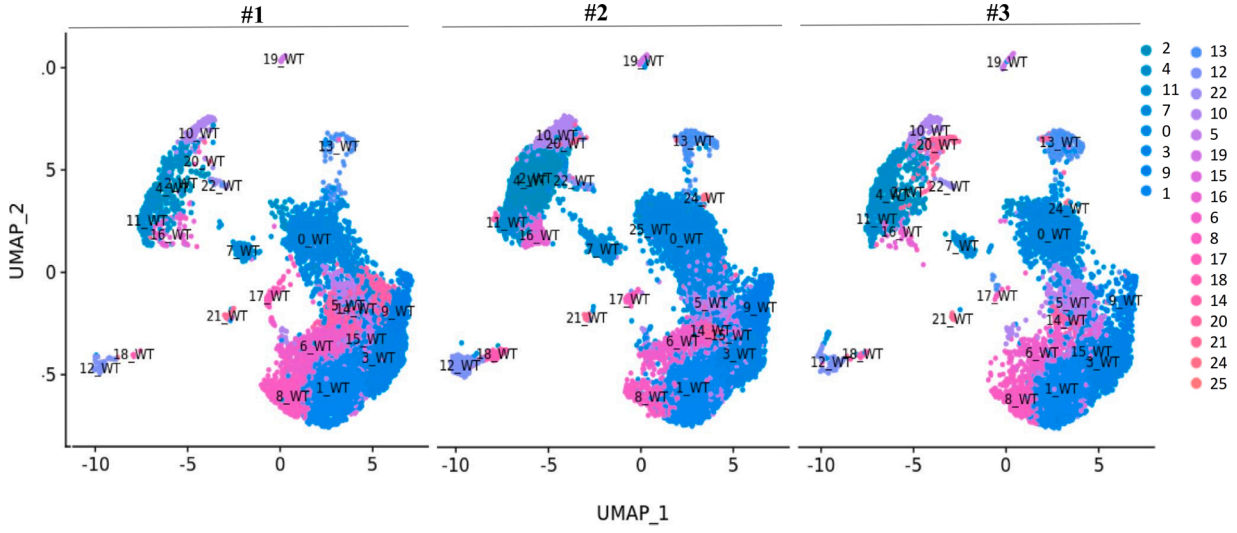

Fig. 3. UMAP visualization of the 25 clusters identified in E15.5 male mouse cortex.
Qualitative evaluation of batch effect correction using UMAP visualization for three datasets from three biological replicates. All clusters are visualized by different colors.

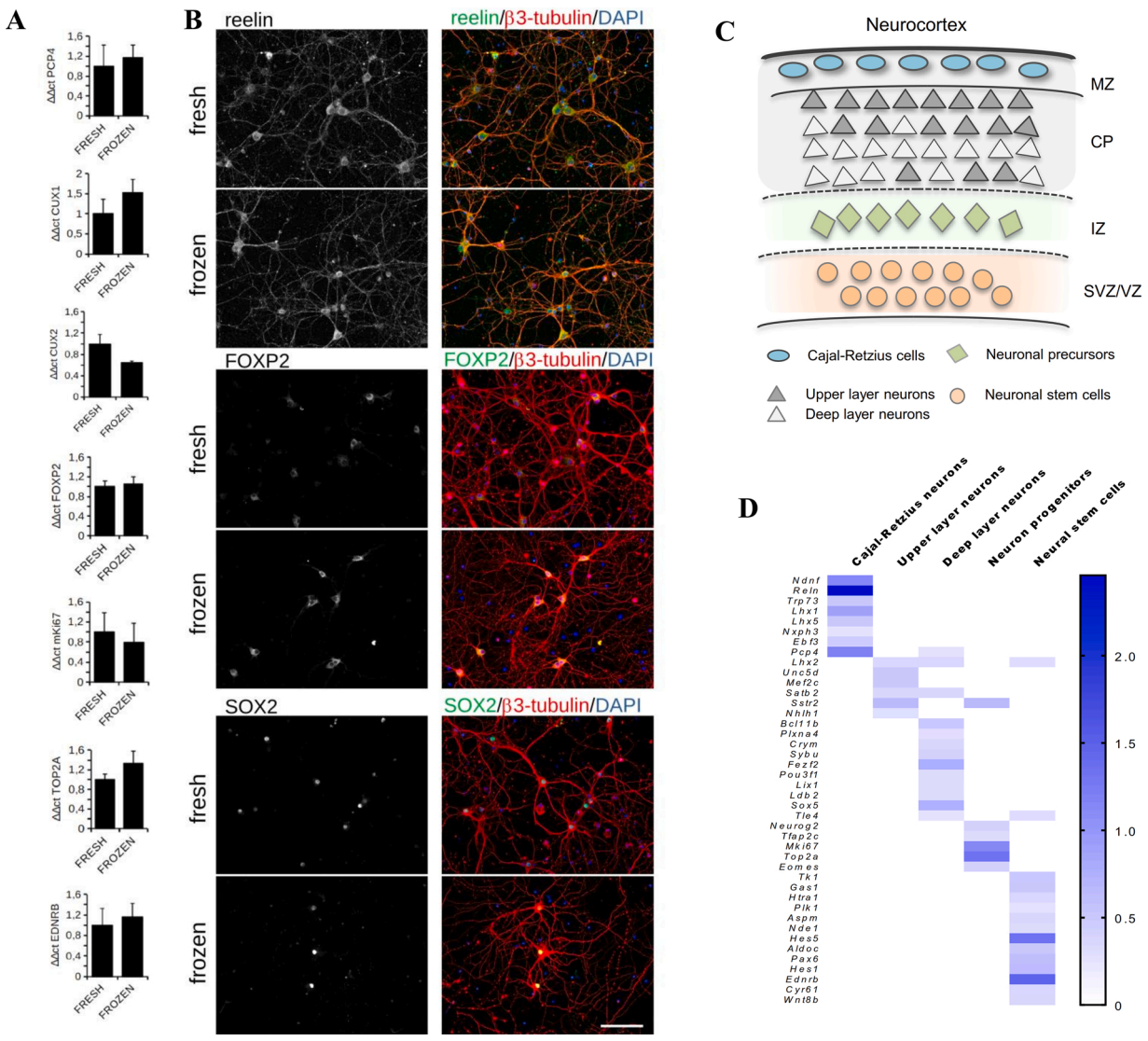

Fig. 4. Prevalence of peculiar neural populations in Neurostore-cryoconserved primary cortical culture. (A) qPCR expression of *Pcp4, Cux1, Cux2*, *Foxp2*, *mKi67*, *Top2A* and *Ednrb* in DIV14 fresh and cryopreserved cortical cultures. Data represent the relative expression in cryopreserved cultures normalized over fresh cultures and are expressed as mean ± S.E. n = 5. *t-test* not significant. (B) Immunofluorescence staining in DIV14 fresh and cryopreserved cultures with antibodies against Reelin, FOXP2, SOX2, and β3-tubulin. Scale bar = 100 μm. (C) A cartoon describing the relative position of each cell sub-type in the neocortex is displayed. Marginal Zone, MZ; Cortical Plate, CP; Intermediate Zone, IZ; Subventricular Zone/Ventricular Zone, SVZ/VZ. (D) A heat map including selected marker genes that distinguish four cell sub-populations in E15.5 cryopreserved cortical cells (Cajal-Retzius cells, upper and deep layer neurons, neuron progenitors and neural stem cells) is shown. Gene expression values range on a scale from white to blue.

similar profiles between 300−700bp and an average around 400bp, obtaining a good technical reproducibility (Fig. S2 *left panels*)**.** The single-cell libraries were sequenced to a median depth of ~32,000 reads/cell with ~330 million reads/experiments, detected a median of ~5500 transcripts per cell and represented a median of ~ 2300 of genes per cell. The total reads, the mean reads/cell, the mean genes/cell, the total genes detected and median transcripts/cell are reported in Table S1 for the three experiments. We found an average of mitochondrial RNAs around 20 % in each sample and we filtered out as described in Methods section, since ribosomal and mitochondrial RNAs are markers of cell apoptosis. Next, we leveraged a non-linear dimensional reduction technique to aggregate transcriptionally similar cells and we removed clusters likely to be of low quality, resulting from debris, doublets/multiplets and dead cells. Our analysis led to the identification of 33,211 cells representing 19 cell populations for WT embryo #1 (Fig. S2A) and #3 (Fig. S2C) and 22 cell populations for WT embryo #2 (Fig. S2B). We also observed differences about the cell number intra clusters and among the three biological replicates (Fig. S2**).** In more detail, clustering analysis identified 19 distinct cell clusters from 18 to 1632 cells/cluster for WT embryo #1, 22 from 99 to 3791 cells/cluster for WT embryo #2 and 19 from 12 to 1162 cells/cluster for WT embryo #3. These numbers are on par or exceeds other similar studies (Loo et al., 2019). Since rare cell types with regulatory roles present subtle transcriptomic signatures and could be obscured by technical or biological confounders, the data integration becomes absolutely necessary. After harmonization analysis, we identified a total of 25 clusters for all the three replicates demonstrating the high reproducibility of our experiments (Fig. 3).

3.3. *Validation of cryopreservation-resuscitation procedure*

We characterized our cultures in terms of the prevalence of peculiar neural populations before and after the cryopreservation-resuscitation procedure. To this aim we extracted the mRNA from DIV14 fresh and cryopreserved cortical cultures and analysed by qPCR the relative expression of gene highly expressed in particular cortical layer (Loo et al., 2019): *Pcp4* (Cajal-Retzius layer), *Cux1* and *Cux2* (upper layer

II-IV), *Foxp2* (deeper layer VI), *mKi67* and *Top2A* (neuronal progenitor), *Ednrb* (neural stem cell). All the genes were equally expressed in fresh and Neurostore-cryoconserved primary cortical cultures (Fig. 4A). Next, we profiled our culture by immunofluorescence means for the expression of Reelin (Cajal-Retzius layer), FOXP2 (deeper layer VI) and SOX2 (neuronal progenitor). The proteins were similarly expressed in fresh and Neurostore-cryoconserved primary cortical culture (Fig. 4B). Furthermore, by scRNAseq experiments we analysed gene expression for a number of selected markers (Fig.S3 and Fig.4C): *Reln, Trp73, Lhx1, Lhx5, Ebf3, Ndnf* and *Pcp4* (Cajal-Retzius layer); *Lhx2, Unc5d, Mef2c, Satb2, Sstr2, Nhlh1, Bcl11b, Plxna4, Crym, Sybu, Fezf2, Pou3f1, Lix1, Ldb2, Sox5* and *Tle4* (cortical layer); *Neurog2, Tfap2c, mKi67, Top2a* and *Eomes* (neuronal progenitors); *Tk1, Gas1, Htra1, Plk1, Aspm, Nde1, Hes5, Aldoc, Pax6, Hes1, Ednrb, Cyr61* and *Wnt8b* (neural stem cells). The expression values of these markers were shown in the heat map to further confirm the applicability of the procedure in scRNASeq study (Fig. 4D)

4. Conclusions

We demonstrated that the Neurostore cryopreservation method is reliable with a scRNASeq experiment, as we validated in mouse primary cortical cells. In addition, expression analysis of cortical markers is consistent with the classical description of cortical layer composition within the normal developing cerebral cortex. Thus, our approach constitutes the starting point for the accurate classification of all cortical sub-types aiming to better understand cellular and molecular processes underlying cortical development in mouse brain. This approach is also powerful and reliable to perform scRNASeq experiments when it is necessary to carry out sample isolation and library preparation in laboratories located in distant places. Furthermore, this method might pave the road also to other potential downstream applications, such as epigenomics or proteomics studies at single cell resolution.

CRediT authorship contribution statement

Lucia Verrillo: Methodology, Data curation, Writing - original draft. **Eleonora Mangano:** Formal analysis, Data curation, Writing - original draft. **Denise Drongitis:** Methodology, Visualization, Investigation, Writing - review & editing. **Ivan Merelli:** Formal analysis. **Francesca Pischedda:** Methodology, Visualization, Writing - review & editing. **Giovanni Piccoli:** Methodology, Visualization, Writing - review & editing. **Clarissa Consolandi:** Investigation, Data curation, Writing - original draft. **Roberta Bordoni:** Investigation, Data curation, Writing - original draft. **Maria Giuseppina Miano:** Writing - original draft, Supervision.

Declaration of Competing Interest

Neurostore is a proprietary formulation developed by G.P. and F.P under patent consideration. The authors are willing to distribute Neurostore media to any colleague interested under a proper MTA.

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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.jneumeth.2020.108960.

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