Medical Research Archives, Volume 4, Issue 5.

Plastic embedding tissue for laser microdissection-assisted developmental genomics analyses at single cell resolution

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ABSTRACT
The analysis of nucleic acids from single cells functioning in their multicellular context is an important part of performing robust developmental genomics studies. Toward this goal, collecting the desired cell(s) from their milieu has been aided by physical means such as laser microdissection. However, tissue processing for laser microdissection has the potential disadvantage of losing molecular information. Procedures involving tissue embedding in plastic have the potential to circumvent these disadvantages, permitting the robust analysis of nucleic acids from single cells isolated from complex tissues.

KEY WORDS:
Technovit 9100; plastic embedding; laser microdissection; pathogen; development; RNA

ABBREVIATIONS:
laser microdissection, LM; plastic embedded laser microdissection, pe-LM; fluorescence activated cell sorting, FACS; optimal cutting temperature, O.C.T.; soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor, SNARE; transmission electron microscopy, TEM; diethyl pyrocarbonate, DEPC

INTRODUCTION
Cell identity is defined by its function. In tissues, each cell has a function that is determined by spatial and temporal cues. Genetic approaches using mutant analyses have aided in our understanding of cell function. However, the downstream effects caused by altered gene function in some cases are left unclear and yet to be understood. Genomics analyses bridge that gap in knowledge. To obtain a faithful understanding of how cells are functioning in their context, it would be useful to isolate them from their multicellular environment so homogeneous samples can be collected for downstream genomics analyses. This goal has been approached in one of three major ways. (1) cell sorting, (2) micromanipulation and (3) laser microdissection (LM). However, in the past the faithful isolation of the desired cells or their cytoplasm to homogeneity has been impeded by the technological limitations of the time. This micro review will introduce each of the three sample collection methods, but focuses primarily on a new approach to laser microdissection that involves the embedding of the tissue in plastic and subsequent nucleic acid isolation.

Cell sorting
Cell sorting is reliant on disconnecting the cells from each other by physical, chemical or enzymatic methods (Fulwyler, 1965). This process is followed by using the physical characteristics of the cells and/or availability of cell type specific molecular markers or antigens to sort the cells. A widely employed procedure used to accomplish this goal is fluorescence activated cell sorting (FACS) or related methodologies (Julius et al. 1972). A unifying feature of the cells used in FACS-based isolation procedures is their ease in separation due to their physical or chemical characteristics. A detailed description is beyond the scope of this work and the reader is directed to a number of papers for details (Fraker et al. 1995; Galbraith et al. 1999; Valitutti and Dessing, 2000; Miura et al. 2000; Herzenberg and De Rosa, 2000; Maric and Barker 2004; Tung et al. 2007; Zhu and Murthy 2013; Schwach and Passier, 2016).

Micromanipulation
Micromanipulation-based methods extract cytoplasm directly from the cell under study. Recent work in the field of neurobiology has shown it is possible to use a patch-clamp device to isolate cytoplasm for downstream analyses (Fuzik et al. 2016; Cadwell et al. 2016). An interesting aspect of this work is that it is

possible to obtain several different types of information from the same cell. For example, the procedure is used to measure cellular morphology and physiology, followed by isolation of the cytoplasm for its use in single cell transcriptomics analyses of that same cell that had been previously analyzed physiologically (Fuzik et al. 2016; Cadwell et al. 2016) The reader is directed to a number of papers for details (Lin et al. 2007; Biase et al. 2014; Fuzik et al. 2016; Cadwell et al. 2016).

Laser microdissection

The LM technology has found use in different biological contexts including the study of DNA mutations, transcriptome, proteome, metabolome and miRNAs (Meier-Ruge et al. 1976; Isenberg et al. 1976; Hedrum et al. 1994; Emmert-Buck et al. 1996, 2000; Bernsen et al. 1998; Schütze & Lahr 1998; Sgroi et al. 1999; Ornstein et al. 2000; Jessani et al. 2002, 2004; Schad et al. 2005; Nonn et al. 2010; Cancer Genome Atlas Research Network 2011; Nishimoto et al. 2012). Furthermore, LM has found much use in plant research to circumvent problems presented by the cell wall that encapsulates the cell of interest (Nakazono et al. 2003; Klink et al. 2005; Matsye et al. 2011). LM is accomplished by embalming the tissue with one of several different types of support media including paraffin or the cryopreservant, optimal cutting temperature (O.C.T.) compound or related mixtures (Goldsworthy et al., 1999; Tam et al., 1999; Bhattacharya et al., 2003; Tadros et al., 2003; Dalmas et al., 2008). The O.C.T and paraffin compounds have their advantages and disadvantages and are described briefly, followed by a description of plastic embedding of tissue for LM which is the focus of this mini review.

Cryogenic embedding tissue for LM

The cryopreservation of tissue is a longstanding method for biological analysis and diagnostics (Cullen, 1895; Wilson, 1905). Out of this approach came the development of O.C.T.,
a material that is composed of polyvinyl alcohol, polyethylene glycol and non-reactive ingredients. Tissue embalmed in O.C.T. has been useful for LM and can be sectioned to thicknesses of at least 10 μm and typically much thicker. However, since the sections are thick the cryopreserved tissues have an increased likelihood that undesired cells or their fragments are also collected. It must be noted that when using O.C.T the ability to obtain thinner sections is temperature-dependent. Therefore, these determinations must be made empirically during the course of the experiment for each tissue. Since the O.C.T. matrix is composed of water soluble glycols and resins that can disrupt membranes, it is possible that the integrity of the tissue is disrupted which would allow cellular contents to be released into the tissue surroundings. This undesirable characteristic of cryopreserved tissue may result in the loss of biological information or mixing of cellular contents within the tissue block or on the slide prior to or after LM. Furthermore, the fine structure of cells is not preserved well when tissue is infiltrated in O.C.T., complicating the identification of specific target cell types. Therefore, while cryopreservation is desirable for limiting the activity of deleterious enzymes such as RNAses which would complicate downstream analyses, the procedure itself may introduce false or misleading structural information in a field of sectioned tissue. These potential difficulties presented by O.C.T. processing methodologies may therefore impose the need for staining, immunostaining or employment of molecular markers to identify the desired cells. This situation is a problem if there are no molecular probes or stains to detect the desired cells since in many cases, the identification of uniquely expressed genes is the reason why the LM technique is used in the first place. These limitations do not mean the use of O.C.T. is detrimental to biological study as it has been used in many analyses for decades (Pusey, 1974; Ishii et al. 1993; Dalmas et al. 2008; Sturm et al. 2013).

Paraffin embedding tissue for LM

A lab pipeline employing paraffin is simple, requiring little technical experience and permits thermal storage indefinitely between 4°C and ambient temperature. In contrast to O.C.T., paraffin has the advantage of preserving anatomical detail well. This characteristic facilitates identification of the desired cells during LM. Undesirable characteristics of paraffin include complications presented in some of the early processing steps involving temperatures that are greater than 50° C prior to casting the tissue which could affect the tissue or stability of mRNA. Tissue blocks can be stored at temperatures as low as 4° C. However, at temperatures below 4° C the paraffin becomes brittle and cracks rendering it useless for study. Another drawback of the paraffin embedding strategy is that the cells must be sectioned at thicknesses of 4 μm or greater because of the physical limitations of the paraffin matrix during sectioning. Regarding transcriptomics, paraffin has the highly undesirable characteristic of being contaminated with RNAses which promotes RNA degradation (Jonsson and Lagerstedt, 1957, 1958; Nair, 1958). Other problems include that the sectioned tissue that is being placed onto microscope slides cannot be visualized directly after sectioning due to the crystalline and opaque nature of paraffin. Therefore, the tissue must be processed through a step that includes a paraffin solvent. This step likely results in the loss of biological molecules into the solvent during processing. After dissolving the paraffin support, the tissue can be observed for LM. However, since there is no mounting medium or cover slip, the light passing through the tissue is refracted in various ways, distorting the image of the tissue passing through the microscope. This problem can make identifying the desired cell or cells difficult or impossible without stains or molecular markers. Even with these limitations, paraffin-mediated LM has permitted a genomics analysis of a plant defense mechanism involving the vesicle transport system that is mediated by the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) (Matsye et al. 2011, 2012; Pant et al. 2014, Sharma et al. 2016).

Plastic embedding tissue for LM

A variation on the paraffin and cryogenic embedding methods is plastic-embedding (pe) the tissue for LM (pe-LM) (Klink et al. 2013; Klink and Thibaudeau, 2014). Plastics have been used for decades in histological analyses for both light and transmission electron microscopy (TEM). The embedding of tissue in plastic has the advantage of preserving fine cellular detail very well. Furthermore, tissue can be processed entirely at low temperatures (-20° C). Depending on the application, the tissue can be ultra-thin sectioned at 0.05–0.1 μm or semi-thin sectioned at thicknesses between 0.1-2 μm. A desirable aspect of embedding the tissue in plastic is that the plastic remains clear after casting and sectioning. This characteristic is unlike paraffin which is opaque after sectioning and O.C.T.-embedded tissue which renders the cells difficult to visualize under the microscope. The clear and usually colorless nature of plastics allows for the visualization of the tissue on the microscope slide directly after semi-thin-sectioning. By definition, since the tissue within the clear, semi-thin sections can be visualized, there is no need to dissolve the plastic prior to LM. Therefore, for the purpose of LM, the plastic itself serves the purpose of a coverslip, preventing refraction of the light as what happens in paraffin and O.C.T-embedded sections.

Choice of plastic

While many different types of plastics exist, one of the best for pe-LM is Technovit® 9100® (Klink et al. 2013, Klink and Thibaudeau, 2014). Technovit® 9100® is a methacrylate resin that is clear and colorless after polymerization. Technovit® 9100® can be cold-polymerized at temperatures between -8 and -20° C with a catalyst. This property of the polymerization

step allows for the dissipation of heat, inhibiting enzymatic activity within the tissue. More importantly for modern molecular analyses, since all tissue processing steps can be done at these low temperatures, RNA quality would be better preserved (Klink et al. 2013; Klink and Thibaudeau, 2014). Technovit® 9100 has been used in many studies including the study of tooth and soft tissues. Technovit 9100 exhibits superior antigen retention as compared to paraffin sections and other plastics (Arnold et al. 1998, 2003; Yang et al. 2003; Brorson and Reinholt 2008; Vertenten et al. 2008; Singhrao et al. 2009; Wittenburg et al. 2009; Steiniger et al. 2013; Bako et al. 2015). The Technovit® 9100®-based pe-LM allows for obtaining semi-thin sections at 0.8 μm, far thinner than the 10 μm or greater typically used for paraffin or cryosectioning and LM (Klink et al. 2013). As stated earlier, a major advantage of the pe-LM procedure is that the plastic does not have to be dissolved away prior to microdissection since the plastic is clear. Therefore, the entire cellular contents can be collected during LM (**Figure 1**). In LM procedures using paraffin as a support matrix, its crystalline and opaque nature interferes with the visualization of the cells. This interference happens because light refracts as it passes through the paraffin and tissue. Therefore, solvents must be used to dissolve the paraffin away prior to the LM procedure. Even then, light is refracted as it encounters the tissue. Therefore, the resolution observed under the microscope of tissue processed in paraffin and O.C.T. is much poorer than tissue processed for pe-LM (Klink et al. 2013; Klink and Thibaudeau, 2014). Furthermore, loss of biological molecules in the form of RNA, microRNAs (miRNAs) proteins and metabolites would be expected to happen as the paraffin is dissolved away and the cytoplasm becomes liberated on the slide (Jonsson and Lagerstedt, 1958; Nair, 1958). In contrast, the collection of serial, semi-thin plastic sections allows for the reconstruction of the entire tissue series onto LM slides. After microdissection, the plastic is dissolved with methanol in the tissue collection tubes and the RNA is isolated by standard LM procedures.

Pe-LM procedure

The pe-LM procedure has been adapted from our previously published methods (Klink et al. 2005, 2007, 2009, 2010a, b, 2011, 2013; Klink and Thibaudeau, 2014; Matsye et al. 2011). The protocol is summarized here. Glassware and metal utensils are made RNAse-free by an 8 hr baking at 180^{o} C. RNAse-free solutions are made with Nano-pure® water (Barnstead, Thermo Fischer Scientific Inc., Waltham, MA) with 0.1% diethyl pyrocarbonate (DEPC) (Sigma, St. Louis, MO). The DEPC is dissolved in aqueous solution and stirred for 12 hr, overnight prior to autoclaving thus removing the DEPC. Tissue fixation occurs in 75% ethanol:25% glacial acetic acid (Klink et al. 2005). Specimen dehydration occurs through a graded ethanol series (75%, 90%, 3 X 100%). Tissue infiltration and embedding using Technovit® 9100® (Electron Microscopy Sciences, Hatfield, PA) with xylene as a transitional fluid is done according to the manufacturer's instructions (http://www.ebsciences.com/histology/methacrylate.htm) (Klink et al. 2013). Tissue pieces are placed into Beem® capsules. Subsequent polymerization occurs at -8^{o} C for 24 hr (Klink et al. 2013). Using a Reichert-Jung Ultracut-E ultramicrotome, semi-thin sections are cut with a diamond knife at 0.8 μm (Leica Microsystems®, Germany). DEPC-treated water is dropped on PEN MembraneSlides® (Leica Microsystems®) set on a slide warmer set at 40° C. This step permits evaporation of the DEPC-treated water so that the plastic sections flatten and adhere to the PEN membrane that coats the slide. Once the water is completely evaporated, the slides are used immediately for LM on an Arcturus® Veritas® microscope (Molecular Devices, Sunnyvale, CA). Cells are collected on CapSure HS LCM Caps (Applied Biosystems, Foster City, CA). Subsequent downstream application

include washing off the cells of the HS cap by micropipetting 20 µl of XB buffer (Applied Biosystems) onto the HS cap. The solution is then moved to a microcentrifuge tube. The RNA samples are then isolated with the PicoPure RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions (Klink et al. 2005, 2007, 2009, 2013). A DNAfree (Ambion) DNAse treatment is done just before the second column wash (Klink et al., 2005, 2007, 2013). Subsequently, RNA yield and quality is determined using the Nanodrop® spectrophotometer (Biorad, Hercules, CA) according to the manufacturer's instructions (Klink et al. 2013; Klink and Thibaudeau, 2014).

CONCLUSION AND FUTURE DIRECTIONS

The application of plastic embedding tissue for laser microdissection is allowing for the collection of cells at unprecedented resolution. This improvement allows for the isolation of biologically relevant molecules for their analysis and placement in the framework of cell physiological and developmental genomics processes. A series of new technologies have been developed for downstream analyses of small amounts of DNA, RNA, protein and metabolites. Because of these technical improvements, the heretofore impediment presented by low quantities of isolated sample is no longer a limitation. These technological improvements therefore are allowing pe-LM to be applied to single cell analyses. In particular, the development of microfluidics is an exciting platform to accomplish high throughput genomics analyses of low quantity sample. The application of pe-LM as a method to obtain RNA and other sample types will undoubtedly be part of the future of single cell biological analyses. Merging small sample preparation approaches to the roust microfluidic arrays will allow for an understanding of cellular processes at high resolution in cells that have been intractable for such study (Jang et al. 2011; Weaver et al. 2014; Trapnell et al. 2014; Pollen et al. 2014, 2015; Xin et al. 2016).

ACKNOWLEDGEMENTS

Start-up support and teaching assistantships from the Department of Biological Sciences are acknowledged. Additional support from the Mississippi Soybean Promotion Board, Cotton Incorporated and Mississippi Agricultural and Forestry Experimental Station (MAFES) is acknowledged. Past graduate students contributing to this project and the genomics analyses from the Department of Biological Sciences are acknowledged. These students include Prachi Matsye, Shankar Pant, Brant McNeece, Keshav Sharma, Prakash Niruala and Tineka Burkhead. The work has been greatly complimented by undergraduate students, including Christina Jones, Suchit Salian, Nishi Sunthwal, Priyanka Gadre, Dollie Welch, Kim Anderson, Brittany Ginn, John Clune, Hannah Burson, Chase Robinson, Meghan Calhoun, Katherine McCracken, Kayla Moore, Madison Milhoan, Erin Curran, Neil Shannon, Austin Martindale, Keigero Fergusen, Ashley Dowdy, Taylor Henry, Hannah Stimson, Hannah Whitlock, Erin Ball, Henry Pittman, Shelby Janeski, Ashlee Vargason, Annedrea McMillan, Courtney Gagliano, Leslie Canale, Alison Antee, Hannah Miller, Adam Crittenden, Lauren Langston, Jamelle Vance, Carolyn Chacon, Emily Carter, Natalie Rentrop, Eileen Modzeleski, Erica Sowell, Chrissy Miller, Anna Bailey Britt, Chelsea Tittle, Robyn Beattie, Dejanie Dilworth, Aishwarya Dikshit, Harshini Sampathkumar, Samantha Rushing, Meagan Young, Kathryn Stiglet, Makenzie Miller, Morgan Urich, Chase Nash, Kyle Winston, Olivia Long, Jody Clark, Ana Simal, Katherine Thrash and Adrienne McMorris. Dr. Gary Lawrence, Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology Mississippi State University, Mississippi State is acknowledged. Dr. Giselle Thibaudeau and Amanda Lawrence, Imaging and Analytical Technologies Center, Mississippi State University are acknowledged. Dr. Wes Burger (MAFES) is acknowledged for his interest.

REFERENCES

1. Arnold WH, Gaengler P, Kalkutschke L. 1998. Three-dimensional reconstruction of approximal subsurface caries lesions in deciduous molars. Clin Oral Investig 2: 174-179

2. Arnold WH, Konopka S, Kriwalsky MS, Gaengler P. 2003. Morphological analysis and chemical content of natural dentin carious lesion zones. Ann Anat 185: 419-424

3. Bako P, Bassiouni M, Eckhard A, Gerlinger I, Frick C, Löwenheim H, Müller M. 2015. Methyl methacrylate embedding to study the morphology and immunohistochemistry of adult guinea pig and mouse cochleae. J Neurosci Methods 254: 86-93

4. Bernsen MR, Dijkman HB, de Vries E, Figdor CG, Ruiter DJ, Adema GJ, van Muijen GN. 1998. Identification of multiple mRNA and DNA sequences from small tissue samples isolated by laser-assisted microdissection. Lab Invest 78: 1267-1273

5. Bhattacharya SH, Gal AA, Murray KK. 2003. Laser capture microdissection MALDI for direct analysis of archival tissue. J Proteome Res 2: 95-98

6. Biase FH, Everts RE, Oliveira R, Santos-Biase WK, Fonseca Merighe GK, Smith LC, Martelli L, Lewin H, Meirelles FV. 2014. Messenger RNAs in metaphase II oocytes correlate with successful embryo development to the blastocyst stage. Zygote 22: 69-79

7. Brorson SH, Reinholt FP. 2008.The intensity of immunogold labeling of deplasticized acrylic sections compared to deplasticized epoxy sections-Theoretical deductions and experimental data. Micron 39: 144-150

8. Cadwell CR, Palasantza A, Jiang X, Berens P, Deng Q, Yilmaz M, Reimer J, Shen S, Bethge M, Tolias KF, Sandberg R, Tolias AS. 2016. Electrophysiological, transcriptomic and morphologic profiling of single neurons using Patch-seq. Nat Biotechnol 34: 199-203

9. Cancer Genome Atlas Research Network. 2011. Integrated genomic analyses of ovarian carcinoma. Nature 474: 609-615

10. Cullen TS. 1895. A rapid method of making permanent sections from frozen sections by the use of formalin. Johns Hopkins Hosp Bull 6: 67

11. Dalmas DA, Scicchitano MS, Chen Y, Kane J, Mirabile R, Schwartz LW, Thomas HC, Boyce RW. 2008. Transcriptional profiling of laser capture microdissected rat arterial elements: fenoldopam-induced vascular toxicity as a model system. Toxicol Pathol 36: 496-519

12. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA. 1996. Laser capture microdissection. Science 274: 998–1001

13. Emmert-Buck MR, Gillespie JW, Paweletz CP, Ornstein DK, Basrur V, Appella E, Wang QH, Huang J, Hu N, Taylor P, Petricoin EF 3rd. 2000. An approach to proteomic analysis of human tumors. Mol Carcinog 27: 158-165

14. Fulwyler MJ. 1965. Electronic separation of biological cells by volume. Science 150: 910–911

15. Fuzik J, Zeisel A, Máté Z, Calvigioni D, Yanagawa Y, Szabó G, Linnarsson S, Harkany T. 2016. Integration of electrophysiological recordings with single-cell RNA-seq data identifies neuronal subtypes. Nat Biotechnol 34: 175-183

16. Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR. 1999. Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. Mol Carcinog 25: 86-91

17. Hedrum A, Pontén F, Ren Z, Lundeberg J, Pontén J, Uhlén M. 1994. Sequence-based analysis of the human p53 gene based on microdissection of tumor biopsy samples. Biotechniques 17, 118-119, 122-124, 126-129

18. Isenberg G., Bielser W., Meier-Ruge W., Remy E. 1976. Cell surgery by laser micro-dissection: a preparative method. J Microsc 107: 19–24

19. Ishii T, Kasama K, Kondo M, Takahash T. 1993. Cryostat Sectioning of Formalin-Fixed Brain: Further Attempt to Improve Section Quality by Previous Infiltration with O.C.T. Compound. Tohoku J Exp Med 171: 101-105

20. Jessani N, Humphrey M, McDonald WH, Niessen S, Masuda K, Gangadharan B, Yates JR 3rd, Mueller BM, Cravatt BF. 2004. Carcinoma and stromal enzyme activity profiles associated with breast tumor growth in vivo. Proc Natl Acad Sci USA 101: 13756–13761

21. Jessani N, Liu Y, Humphrey M, Cravatt BF. 2002. Enyzme activity profiles of the secreted and membrane proteome that depict cancer cell invasiveness. Proc Natl Acad Sci USA 99: 10335–10340

22. Jang JS, Simon VA, Feddersen RM, Rakhshan F, Schultz DA, Zschunke MA, Lingle WL, Kolbert CP, Jen J. 2011. Quantitative miRNA expression analysis using fluidigm microfluidics dynamic arrays. BMC Genomics 12: 144. doi: 10.1186/1471-2164-12-144

23. Jonsson N, Lagerstedt S. 1957. Demonstration of ribonuclease activity in sections from carnoy fixed rat pancreas. Experientia 13: 321-323

24. Jonsson N, Lagerstedt S. 1958. Losses of nucleic acid derivatives from fixed tissue during flattening of paraffin sections on water. Experientia 14: 157-159

25. Julius MH, Masuda T, Herzenberg LA. 1972. Demonstration that antigen-binding cells are precursors of antibody-producing cells after purification with a fluorescence-activated cell sorter. Proc Natl Acad Sci USA 69: 1934–1938

26. Klink VP, Hosseini P, Matsye PD, Alkharouf NW, Matthews BF. 2011. Differences in gene expression amplitude overlie a conserved transcriptomic program occurring between the rapid and potent localized resistant reaction at the syncytium of the *Glycine max* genotype Peking (PI 548402) as compared to the prolonged and potent resistant reaction of PI 88788. Plant Molecular Biology 75: 141-165

27. Klink VP, Overall CC, Alkharouf NW, MacDonald MH, Matthews BF. 2010b. Microarray detection calls as a means to compare transcripts expressed within syncytial cells isolated from incompatible and compatible soybean (Glycine max) roots infected by the soybean cyst nematode (Heterodera glycines). Journal of Biomedicine and Biotechnology 1-30

28. Klink VP, Hosseini P, Matsye PD, Alkharouf NW, Matthews BF. 2010a Syncytium gene expression in *Glycine max*$_{[PI\ 88788]}$ roots undergoing a resistant reaction to the parasitic nematode *Heterodera glycines*. Plant Physiology and Biochemistry 48: 176-193

29. Klink VP, Hosseini P, Matsye PD, Alkharouf NW, Matthews BF. 2009. A gene expression analysis of syncytia laser microdissected from the roots of the *Glycine max* (soybean) genotype PI 548402 (Peking) undergoing a resistant reaction after infection by *Heterodera glycines* (soybean cyst nematode). Plant Molecular Biology 71: 525-567

30. Klink VP, Overall CC, Alkharouf N, MacDonald MH, Matthews BF. 2007. Laser capture microdissection (LCM) and comparative microarray expression analysis of syncytial cells isolated from incompatible and compatible soybean roots infected by soybean cyst nematode (Heterodera glycines). Planta 226: 1389-1409

31. Klink VP, Alkharouf N, MacDonald M, Matthews BF. 2005. Laser capture microdissection (LCM) and analysis of Glycine max (soybean) syncytial cells formed by the soybean cyst nematode Heterodera glycines. Plant Molecular Biology 59: 969-983

32. Lin DM, Loveall B, Ewer J, Deitcher DL, Sucher NJ. 2007. Characterization of mRNA expression in single neurons. Methods Mol Biol 399: 133-152

33. Matsye PD, Kumar R, Hosseini P, Jones CM, Tremblay A, Alkharouf NW, Matthews BF, Klink VP. 2011. Mapping cell fate decisions that occur during soybean defense responses. Plant Molecular Biology 77: 513-528

34. Matsye PD, Lawrence GW, Youssef RM, Kim K-H, Matthews BF, Lawrence KS, Klink VP. 2012. The expression of a naturally occurring, truncated allele of an □-SNAP gene suppresses plant parasitic nematode infection. Plant Molecular Biology 80: 131-155

35. Meier-Ruge W, Bielser W, Remy E, Hillenkamp F, Nitsche R, Unsold R. 1976. The laser in the Lowry technique for microdissection of freeze-dried tissue slices. Histochem J 8: 387-401

36. Nakazono M, Qiu F, Borsuk LA, Schnable PS. 2003. Laser-capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: identification of genes expressed differentially in epidermal cells or vascular tissues of maize. Plant Cell. 15: 583–596

37. Nair KK. 1958. The effect of some common fixatives on the enzymatic activity of ribonuclease. Experientia 14: 172-173

38. Nishimoto K, Rigsby CS, Wang T, Mukai K, Gomez-Sanchez CE, Rainey WE, Seki T. 2012. Transcriptome analysis reveals differentially expressed transcripts in rat adrenal zona glomerulosa and zona fasciculata. Endocrinology 153: 1755-1763

39. Nonn L, Vaishnav A, Gallagher L, Gann PH. 2010. mRNA and micro-RNA expression analysis in laser-capture microdissected prostate biopsies: valuable tool for risk assessment and prevention trials. Exp Mol Pathol 88: 45-51

40. Ornstein DK, Gillespie JW, Paweletz CP, Duray PH, Herring J, Vocke CD, Topalian SL, Bostwick DG, Linehan WM, Petricoin EF 3rd, Emmert-Buck MR. 2000. Proteomic analysis of laser capture microdissected human prostate cancer and in vitro prostate cell lines. Electrophoresis 21: 2235-2242

41. Pant SR, Matsye PD, McNeece BT, Sharma K, Krishnavajhala A, Lawrence GW**,** Klink VP. 2014. Syntaxin 31 functions in *Glycine max* resistance to the plant parasitic nematode *Heterodera glycines* Plant Molecular Biology 85: 107–121

42. Pollen AA, Nowakowski TJ, Shuga J, Wang X, Leyrat AA, Lui JH, Li N^3,

Szpankowski L, Fowler B, Chen P, Ramalingam N, Sun G, Thu M, Norris M, Lebofsky R, Toppani D, Kemp DW 2nd, Wong M, Clerkson B, Jones BN, Wu S, Knutsson L, Alvarado B, Wang J, Weaver LS, May AP, Jones RC, Unger MA, Kriegstein AR, West JA. 2014. Low-coverage single-cell mRNA sequencing reveals cellular heterogeneity and activated signaling pathways in developing cerebral cortex. Nat Biotechnol 32: 1053-1058

43. Pollen AA, Nowakowski TJ, Chen J, Retallack H, Sandoval-Espinosa C, Nicholas CR, Shuga J, Liu SJ, Oldham MC, Diaz A, Lim DA, Leyrat AA, West JA, Kriegstein AR. 2015. Molecular identity of human outer radial glia during cortical development. Cell 163: 55-67

44. Pusey W. 1974. Paraffin embedding after O.C.T. Stain Technol 49: 410-11

45. Schad M, Mungur R, Fiehn O, Kehr J. 2005. Metabolic profiling of laser microdissected vascular bundles of Arabidopsis thaliana. Plant Methods 1: 2

46. Schütze K, Lahr G. 1998. Identification of expressed genes by laser-mediated manipulation of single cells. Nat Biotechnol 16: 737-742

47. Schwach V, Passier R. 2016. Generation and purification of human stem cell-derived cardiomyocytes. Differentiation 91: 126-138

48. Sgroi DC, Teng S, Robinson G, LeVangie R, Hudson JR Jr, Elkahloun AG. 1999. In vivo gene expression profile analysis of human breast cancer progression. Cancer Res 59: 5656-5661

49. Sharma K, Pant SR, McNeece BT, Lawrence GW, Klink VP. 2016. Co-regulation of the *Glycine max* soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE)-containing regulon occurs during defense to a root pathogen. Journal of Plant Interactions 11: 74–93

50. Singhrao SK, Müller CT, Gilbert SJ, Duance VC, Archer CW. 2009. An immunofluorescence method for postembedded tissue in the acrylic resin Technovit 9100 New using fluorescein isothiocyanate secondary detection. Microsc Res Tech 72: 501-506

51. Steiniger BS, Bubel S, Böckler W, Lampp K, Seiler A, Jablonski B, Guthe M, Stachniss V. 2013. Immunostaining of Pulpal Nerve Fibre Bundle/Arteriole Associations in Ground Serial Sections of Whole Human Teeth Embedded in Technovit® 9100. Cells Tissues Organs 198: 57-65

52. Sturm D, Marselli L, Ehehalt F, Richter D, Distler M, Kersting S, Grützmann R, Bokvist K, Froguel P, Liechti R, Jörns A, Meda P, Baretton GB, Saeger HD, Schulte AM, Marchetti P, Solimena M. 2013. Improved protocol for laser microdissection of human pancreatic islets from surgical specimens. J Vis Exp 2013 Jan 6;(71). pii: 50231 doi:10.3791/50231

53. Tadros Y, Ruiz-Deya G, Crawford BE, Thomas R, Abdel-Mageed AB. 2003. In vivo proteomic analysis of cytokine expression in laser capture-microdissected urothelial cells of obstructed ureteropelvic junction procured by laparoscopic dismembered pyeloplasty. J Endourol 17: 333-336

54. Tam AS, Foley JF, Devereux TR, Maronpot RR Massey TE. 1999. High frequency and heterogeneous distribution of p53 mutations in aflatoxin B1-induced mouse lung tumors. Cancer Res 59: 3634-3640

55. Weaver JM, Ross-Innes CS, Shannon N, Lynch AG, Forshew T, Barbera M, Murtaza M, Ong CA, Lao-Sirieix P, Dunning MJ, Smith L, Smith ML, Anderson CL, Carvalho B,

O'Donovan M, Underwood TJ, May AP, Grehan N, Hardwick R, Davies J, Oloumi A, Aparicio S, Caldas C, Eldridge MD, Edwards PA, Rosenfeld N, Tavaré S, Fitzgerald RC; OCCAMS Consortium. 2014. Ordering of mutations in preinvasive disease stages of esophageal carcinogenesis. Nat Genet 46: 837-843

56. Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, Lennon NJ, Livak KJ, Mikkelsen TS, Rinn JL. 2014. The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. Nat Biotechnol 32: 381-386

57. Vertenten G, Vlaminck L, Ducatelle R, Lippens E, Cornelissen M, Gasthuys F. 2008. Immunohistochemical analysis of low-temperature methylmethacrylate resin-embedded goat tissues. Anat Histol Embryol 37: 452-457

58. Wilson LB. 1905. A method for the rapid preparation of fresh tissues for the microscope. *JAMA* 45: 1737

59. Wittenburg G, Volkel C, Mai R, Lauer G. 2009. Immunohistochemical comparison of differentiation markers on paraffin and plastic embedded human bone samples. J Physiol Pharmacol 60 Suppl 8: 43-49

60. Yang R, Davies CM, Archer CW, Richards RG. 2003. Immunohistochemistry of matrix markers in Technovit 9100 New-embedded undecalcified bone sections. Eur Cell Mater 6: 57-71

61. Xin Y, Kim J, Ni M, Wei Y, Okamoto H, Lee J, Adler C, Cavino K, Murphy AJ, Yancopoulos GD, Lin HC, Gromada J. 2016. Use of the Fluidigm C1 platform for RNA sequencing of single mouse pancreatic islet cells. Proc Natl Acad Sci USA 113: 3293-3298

FIGURES

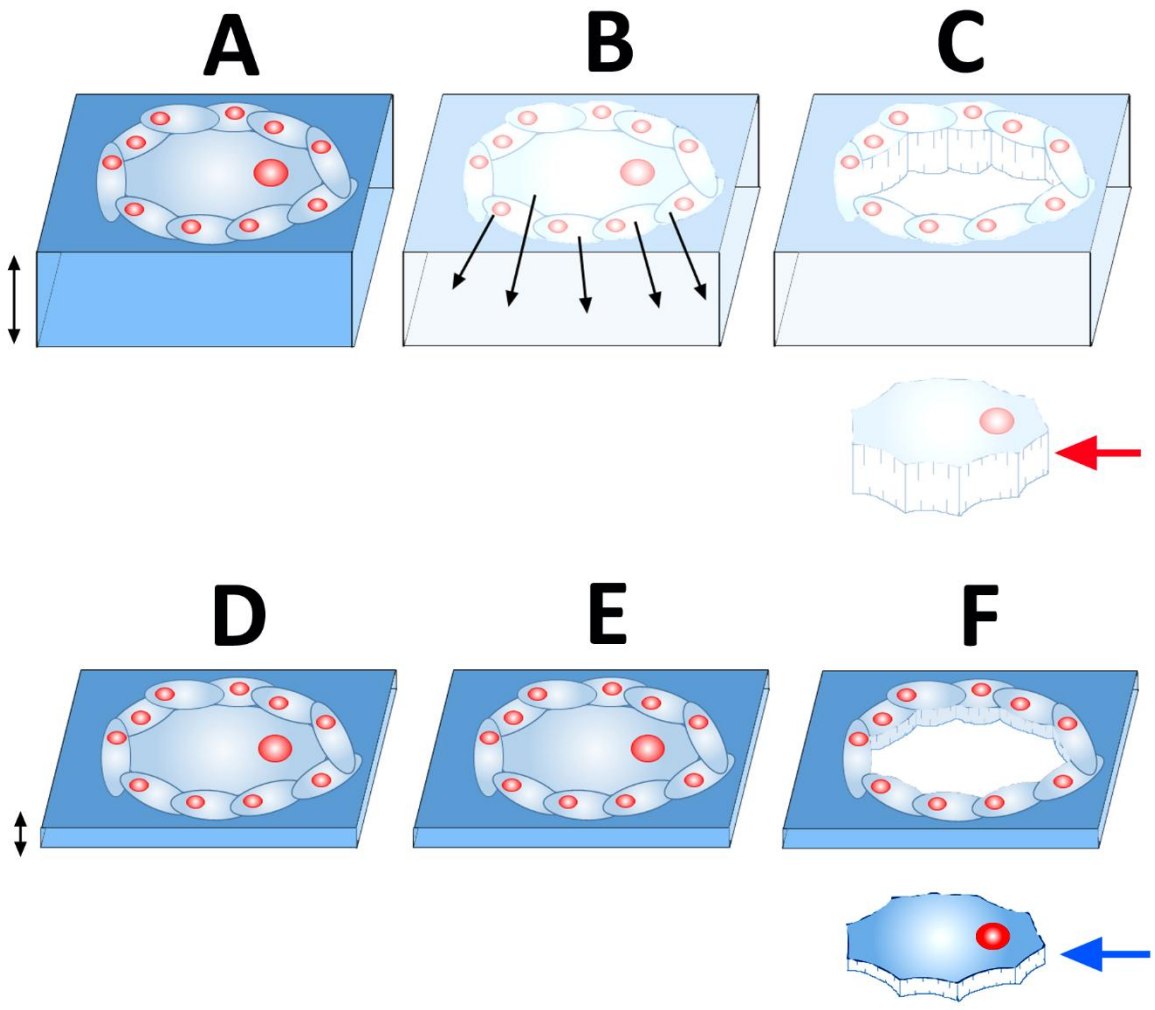

Figure 1.
The advantage of pe-LM, comparing paraffin (**A-C**) and plastic (**D-F**) embedding approaches. **A**, tissue, represented in light blue with a red nucleus is illustrated in the paraffin support (dark blue box). The black vertical arrow (left) represents a 4 µm section limit. **B**, paraffin is dissolved away, accompanied by the loss of biological information (i.e. RNA) that is represented by the lighter hues of the cell and nucleus with the black arrows showing loss of molecules out of the cell. **C**, the cell (red arrow) after LM. **D**, tissue, represented in light blue with a red nucleus is illustrated in the plastic support (dark blue box). The black vertical arrow (left) represents a 0.8 µm semi-thin section. **E**, there is no loss of biological

molecules since the plastic does not have to be etched away because both visualization and sectioning can be done effectively without that step. **F**, pe-LM of a semi-thin section of tissue containing the target cell.

An advantage of the method is that the pe-LM procedure vaporizes neighboring cells. This facet maximizes the likelihood of collecting the desired cell (blue arrow).