

Optimal Molecular Profiling of Tissue and Tissue Components

*Defining the Best Processing and Microdissection
Methods for Biomedical Applications*

**G. Steven Bova, Isam A. Eltoum, John A. Kiernan,
Gene P. Siegal, Andra R. Frost, Carolyn J. M. Best,
John W. Gillespie, and Michael R. Emmert-Buck**

Summary

Isolation of well-preserved pure cell populations is a prerequisite for sound studies of the molecular basis of pancreatic malignancy and other biological phenomena. This chapter reviews current methods for obtaining anatomically specific signals from molecules isolated from tissues, a basic requirement for productive linking of phenotype and genotype. The quality of samples isolated from tissue and used for molecular analysis is often glossed-over or omitted from publications, making interpretation and replication of data difficult or impossible. Fortunately, recently developed techniques allow life scientists to better document and control the quality of samples used for a given assay, creating a foundation for improvement in this area. Tissue processing for molecular studies usually involves some or all of the following steps: tissue collection, gross dissection/identification, fixation, processing/embedding, storage/archiving, sectioning, staining, microdissection/annotation, and pure analyte labeling/identification. High-quality tissue microdissection does not necessarily mean high-quality samples to analyze. The quality of biomaterials obtained for analysis is highly dependent on steps upstream and downstream from tissue microdissection. We provide protocols for each of these steps, and encourage you to improve upon these. It is worth the effort of every laboratory to optimize and document its technique at each stage of the process, and we provide a starting point for those willing to spend the time to optimize. In our view, poor documentation of tissue and cell type of origin and the use of nonoptimized protocols is a source of inefficiency in current life science research. Even incremental improvement in this area will increase productivity significantly.

Key Words: Molecular profiling; tissue processing; tissue staining; sample processing; laser microdissection; RNA; DNA; quality control; workflow management.

1. Introduction

Isolation of well-preserved pure cell populations is a prerequisite for sound studies of the molecular basis of pancreatic malignancy and other biological phenomena. This chapter reviews current methods for obtaining anatomically specific signals from molecules isolated from tissues, a basic requirement for productive linking of phenotype and genotype.

The quality of samples isolated from tissue and used for molecular analysis is often glossed over or omitted from publications, making interpretation and replication of data difficult or impossible. Fortunately, recently developed techniques allow life scientists to better document and control the quality of samples used for a given assay, creating a foundation for improvement in this area.

Tissue processing for molecular studies usually involves some or all of the steps identified in **Fig. 1**. This diagram will serve as a guide for the remainder of the discussion in this chapter.

Great tissue microdissection does not necessarily mean great samples to analyze. The quality of biomaterials obtained for analysis is highly dependent on steps upstream and downstream from tissue microdissection. It is worth the effort of every laboratory to optimize and document its technique at each stage of the process. Isolation of molecular materials from tissue components is a field in rapid evolution, and creativity in developing better ways to obtain pure cell populations and pure components is needed. In our view, poor documentation of tissue and cell type of origin and the use of nonoptimized protocols is a source of inefficiency in current life science research. Even incremental improvement in this area will increase productivity significantly.

Most of the discussion in this chapter refers to cells in solid tissues; it applies equally to cells from body fluids or tissue aspirates when these cells are placed on glass slides or membrane-coated slides for microdissection. Flow cytometric cell purification is not discussed in detail here, but should be considered as an alternative to microdissection techniques whenever intact cells or cell components can be conveniently disaggregated and flow-separated based on reliable immunostaining or other features.

Before starting a study requiring isolated cells or cell components, it is wise to consider the following:

- What biomolecules (DNA, RNA, protein, carbohydrate, lipid) need to be recovered, how much is needed, and what level of purity is acceptable? Preliminary experiments may be needed to define how much starting material is needed and how pure the samples need to be.
- What is the required starting condition of the tissues to be dissected? If the tissue is frozen, how soon after loss of blood perfusion (loss of blood supply and/or nutrient supply) will it be frozen, and will the delay between perfusion loss and freezing/chem-

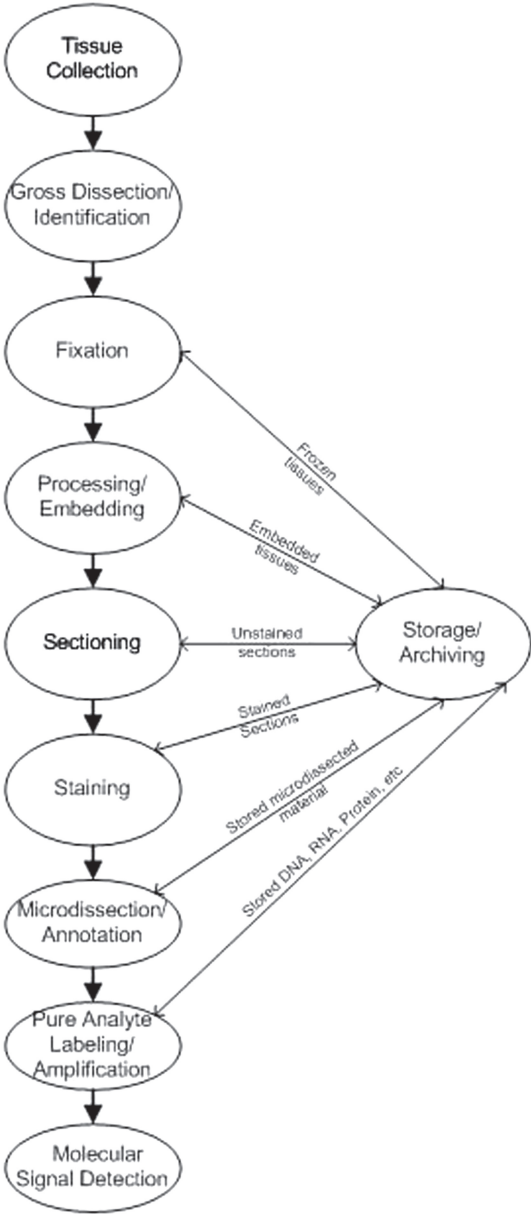


Fig. 1. Tissue processing for molecular analysis: flow diagram.

ical fixation affect the biomolecules you seek to examine? Will drugs that the tissue donor has received affect the molecules to be analyzed within the tissue? Does the tissue have a high level of endogenous or exogenous bacterial or fungal DNase or

RNase activity? Will frozen sections provide adequate histological detail to allow dissection of the cells of interest? If the tissue was chemically fixed (denoted “fixed” in the remainder of this chapter), are the target biomolecules in acceptable condition for the planned assay? For example, synthesis of full-length cDNA would be unlikely from formalin-fixed tissues, and target structures may not be adequately visualized from frozen sections, depending on how the structures are to be identified, and how the tissue sample was frozen.

- What tissue dissection method will be most verifiable (i.e., providing evidence that the cells targeted were actually obtained without contamination), efficient, economical, and best documented?

Combining detailed answers to these questions with informed selection from the various options discussed in this chapter will optimize molecular profiling productivity.

The following information is based on methods currently in use in our laboratories. However, it is not meant to be encyclopedic and the cited references provide a good starting point for additional reading. Also note that the history of tissue fixation and microdissection is not covered here. A brief review of this history is contained in a recent review by Eltoum et al. (*1*) and is touched on by Srinivasan et al. (*2*), and some of the key molecular methods discussed here (and additional topics) are also detailed in articles from the NCI Laboratory of Pathology Pathogenetics Unit (*3–10*). Some of the useful textbooks in the field are also listed in the bibliography (*11–14*). The books by Kiernan (*13*, *14*) provide valuable information on chemical changes induced by fixation and staining that may be useful for those wishing to design and test new protocols. An e-mail listserver for broadcasting specific histotechnology-related questions (with a searchable archive of past questions) is also available (*15*).

1.1. Biosafety Issues

Be sure to consider biosafety needs related to tissue handling in your laboratory. Tissues should always be handled using Universal Precautions. Fresh or processed tissues or their components should not come in direct contact with skin or mucous membranes, and in situations where tissue components could be released in the air, ventilatory isolation (by wearing masks and using biosafety hoods or other containment devices) should be used. Immunizations for preventable infections such as hepatitis B should be considered if the risk of exposure is considered significant. Higher levels of isolation are required if exposure to tuberculosis, prion disease, or other infectious disease is likely. If the tissues studied could contain particularly toxic substances (radioactive isotopes, chemotherapy drugs, etc.), appropriate steps should be taken to prevent significant exposure by laboratory personnel.

1.2. Tissue Collection and Processing

The best tissue collection method will depend on the specifics of your situation. Interval between loss of blood perfusion and cooling or fixation, method of fixation, and uniformity of labeling and processing methods are some of the critical parameters for any study and are discussed in more detail below.

“Fixation” of tissues can occur through freezing and/or through chemical fixation. We discuss only formaldehyde fixation (because it is still the standard) and alcohol-based tissue fixation (because it is a simple and inexpensive alternative that has worked in our hands), but the reader should be aware that a number of proprietary fixatives that claim to provide good histologic and molecular preservation are also available.

1.3. Staining of Tissue Sections

Hematoxylin and eosin (H&E) staining has been the standard diagnostic tissue section staining method for more than a century. For molecular analyses, under the right conditions (*see Subheading 3.*), DNA and RNA can be obtained from H&E-stained material. Hematoxylin stains negatively charged molecules including nucleic acids and rough endoplasmic reticulum blue-violet, and eosin stains positively charged moieties including positively charged amino acids pink-red. Eosins are halogenated derivatives of fluorescein, and eosin Y is the form of eosin in most common use. Both RNA and DNA can be isolated from H&E-stained sections, if the tissue is well preserved and stained properly. Because it fluoresces, eosin interferes with many protein analyses using fluorescent detection. It should also be noted that Mayer’s hematoxylin itself does not stain tissue. In solution, oxidizing agents such as alum ($\text{AlK}[\text{SO}_4]_2 \cdot 12\text{H}_2\text{O}$) convert hematoxylin to hematein. The correct terminology for this stain is Mayer’s hemalum, which is a concatenated product of hematein and alum. Other types of hematoxylin use other oxidizing agents.

Methylene blue is a cationic dye. It stains DNA, RNA, and carbohydrate polyanions. Cytoplasm is strongly stained if a cell is rich in RNA (neurons with Nissl substance, secretory cells, etc.) or anionic mucosubstances (heparin in mast cells; many types of mucous). It is used prior to microdissection for DNA and protein isolation, but not for RNA isolation (*16*).

Methyl green stains nuclei dark green, cytoplasm light green. According to a credible but nonpeer-reviewed study by Agilent, methyl green was best for RNA isolation when compared to the other stains mentioned here (*16*); methyl green is also reportedly compatible with DNA and protein isolation. Please note that what is currently sold as “methyl green” is actually “ethyl green” chemically. True “methyl green” has not been available for about 30 yr (*17*).

Nuclear fast red stains nuclei dark red, cytoplasm lighter red. It is always used in conjunction with an aluminum salt, and its mechanism of action is not known. It is important to buy the right dye (Chemical Index or CI 60760) because the same name is sometimes used to label on other dyes that will not work (17). In the same Agilent study, nuclear fast red performed as well as H&E for RNA isolation, but better than methylene blue.

1.3.1. Immunostaining for Microdissection

Tissue microdissection for molecular analysis is frequently limited by the difficulty in identifying cell types and structures by morphology combined with tinctorial (e.g., H&E) staining alone. The NCI Laboratory of Pathology Pathogenetics Unit and others have developed rapid immunostaining procedures for microdissection and RNA extraction from frozen sections (6), as summarized here. This method allows mRNA analysis of specific cell populations that have been isolated according to immunophenotype. Sections fixed in acetone, methanol, or ethanol/acetone give excellent immunostaining after only 12–25 min total processing time. Specificity, precision, and speed of microdissection are markedly increased due to improved identification of desired cell types.

1.4. Preparation of Cytologic Specimens for Microdissection

Cells centrifuged from body fluids or fine-needle aspirates, or cells propagated in vitro can be prepared for microdissection by making direct smears or through a number of effective proprietary methods for creating thin layers of cells in designated areas of microscope slides. A subset of less adherent cells within fresh tissues can also be rapidly sampled by gentle scraping with a scalpel blade and then rapidly spreading the scraped sample onto a glass slide with the blade. The choice of strategy for preparing cell suspensions for microdissection depends on the anticipated cellularity of the sample. Highly cellular samples can be prepared as direct smears and effectively utilized for laser microdissection, less cellular samples can be concentrated using one of the proprietary cell concentration methodologies such as Cytospin® (Thermo Shandon Inc.), or more recent technologies such as ThinPrep (Cytoc Corp.) or AutocytePrep (TriPath Imaging).

1.5. Manual Microdissection of Blocks and Slides

1.5.1. Cryostat-Based Manual Dissection of Frozen Tissue Blocks

It is often possible to obtain sufficient purity and relatively prodigious quantities of DNA, RNA, or protein from serial manual dissection of frozen tissue blocks directly on a suitable cryostat. Below we describe a method that can

increase purity from 10–50% to 75–95% for cell types that grow in macroscopic clusters.

1.5.2. Manual Microdissection of Tissue Sections on Slides

Several manual microdissection methods can be performed on glass slides, and innovation in manual microdissection methods has continued despite the recent development of laser-based microdissection approaches. Techniques using hand-held tools (18), mechanical micromanipulators (19), manually cutting out areas of sections mounted on cellophane tape (20), ultrasonic oscillating needles (21), and methods specific to cytology specimens (22,23), have been described. Along these lines, Eppendorf has recently marketed a “Piezo Power Micro Dissection (PPMD) System” that is inexpensive relative to the laser dissection systems, and may work well when small quantities of dissected material are needed. The advantage of manual dissection is simpler equipment requirements, making it accessible to most laboratories. Its disadvantages are as follows: it is time consuming; it has a steep learning curve; the smallest dissectable region of interest (ROI) is generally significantly larger than that routinely obtainable with laser-based approaches; and documentation of manual dissection is usually not of as high a quality because it does not fit easily into the manual microdissection workflow.

1.6. Laser-Based Tissue Microdissection Systems

Arcturus, Leica, and Zeiss/PALM laser-based Tissue Section Microdissection Systems are discussed here. All three systems are effective depending on specific needs of the user, and each instrument has its advantages and disadvantages. A comparison of the Arcturus, Leica, and Zeiss/PALM systems is contained in **Table 1**. Laser tissue microdissection systems have also recently been made available by Bio-Rad (Hercules, CA) and MMI AG (Glottbrugg, Switzerland), neither of which is discussed here.

1.6.1. Arcturus PixCell Ite LCM (Laser Capture Microdissection) System

LCM utilizes an infrared laser integrated into a standard inverted microscope, and is based on patented “Laser Capture Microdissection” (LCM) technology originally described by Emmert-Buck et al. (24), and licensed to Arcturus Inc. (Santa Clara, CA, USA). Arcturus introduced its first PixCell system based on this technology in 1996. In LCM, a transparent plastic (CapSureTM, Arcturus, Mountain View, CA, USA) cap with attached ethylene vinyl acetate (EVA) transparent thermoplastic membrane is placed on the surface of a non-cover-slipped, stained tissue section mounted on a standard glass slide. The EVA film is in direct contact (CapSure Macro caps) or slightly above (CapSure HS caps) the tissue section (**Fig. 2**). CapSure HS caps are designed to reduce or eliminate the

Table 1
Comparison of Critical Features of Arcturus, Leica, and PALM Tissue Microdissection Systems Available in 2003

	Arcturus PixCell IIe	Leica AS LMD	PALM Micro Beam
Tissue Isolation Method(s)	(a) User melts EVA (ethylene vinyl acetate) plastic onto ROI using IR laser (980–1064 nm), and ROI remains attached to EVA, while remainder of tissue remains on slide.	(a) UV-A Laser (337 nm) cutting of circumscribed ROI from inverted membrane-coated slides and cover slips. (b) “Cold” ablation of undesired areas with UV-A laser (337 nm)	(a) Noncontact transfer of ROI from slide surface by laser pressure catapulting (LPC) (b) “Cold” ablation of undesired areas with UV-A laser (337 nm) (c) UV-A laser cutting of circumscribed ROI from membrane-coated slides and cover slips, followed by laser pressure catapulting of selected membrane region
ROI:			
Region of interest			
Selection of ROI	Manual control of stage motion linked to video image or direct observation through microscope.	Automated optically-controlled laser beam targeting controlled by user selection of ROI on computer screen (neither stage nor laser moves). User can also view through eyepieces. User can select multiple ROIs within one field at a time.	Automated stage motion (stepping size 128 nm) controlled by user selection of ROI on computer screen. User can select multiple coded ROIs within and outside initial image followed by automated collection process.

Visualization of ROI	Through eyepieces or on video or computer screen. $\times 4$ up to $\times 40$ objectives only, cannot use $\times 100$ objective (working distance limitation). Because of refractive index mismatch, visualization is often difficult or inadequate in regular use, depending on cells desired and tissue background.	Through eyepieces or on computer screen. Leica recently improved manufacture of film-coated slides, improving visualization of tissue.	Visualization on computer screen. In a demo, visualization decreased on membrane, and similar to Arcturus on regular slide, but massive improvement in visualization when dilute sterile mineral oil "liquid cover slip" placed on tissue. Selected ROIs color coded on computer screen.
Lower size limit of a circular ROI	Theoretically, if plastic film adhesion is sufficient, can go to $5\text{ }\mu\text{m}$ or less. In practice, cells in tissues often will not be removed with only a $5\text{-}\mu\text{m}$ diameter circular spot, because adhesion to the slide is greater than adhesion to the plastic film. Dissection of organelles not feasible.	At X66 high dry magnification, able to dissect $5\text{-}\mu\text{m}$ diameter area without difficulty. X100 not available on model tested, but reportedly can be done on coated cover slip.	Depending on magnification and N.A. of objective. $<1\text{ }\mu\text{m}$ diameter cutting can be routinely achieved with X100 1.3 N.A. objective. Dissection of organelles claimed by manufacturer, but not tested.
Disposables	Required use of original CapSure® LCM Macro Cap or CapSure® LCM HS Cap and associated materials	Required use of membrane-coated slides. Can purchase from Leica, or can make your own with superglue and film roll purchased from Leica.	Can use device without proprietary disposables. Optional use of system with proprietary PALM membrane coated slides and coverslips.

(continued)

Table 1 (Continued)

	Arcturus PixCell IIe	Leica AS LMD	PALM Micro Beam
Service history	Simpler device than the other two considered. IR laser is solid state, should never need service. History of excellent service in the United States.	No firsthand or anecdotal information available, but should be handled by Leica technicians. Pulsed nitrogen laser estimated good for 2 million pulses (approx 2 years).	No firsthand or anecdotal experience available. Pulsed Nitrogen laser estimated good for 2 million pulses (approx 2 years), but is covered by service contract. Service will be handled by fully PALM trained Carl Zeiss USA technicians.
Fluorescence option	Yes, does allow image light integration.	Yes. No information on integration of light.	Yes. Currently does not allow image light integration, but software modification to allow this is in pipeline.
Can fully dissect samples greater than 20 microns thick in a single pass?	No. Adhesive property of cap film not sufficient to pull off tissue.	Yes. Useful if tissue is homogeneous.	Yes. Useful if tissue is homogeneous.
Software and documentation of dissection	Documentation of images as desired by user during dissection. Documentation of laser settings and images taken recorded in job file written to hard drive.	Standard software allows documentation of images only. Image database upgrade allows all electronic parameters to be recorded. Database can be linked to other software.	Documentation of images as desired by user during dissection. Key laser and slide position settings also stored automatically.

Quality of dissected materials for molecular analysis	Numerous publications illustrating isolation of various cellular components using LCM. Because dissection is contact-based, care must be taken to avoid contamination of cap with unwanted material; this can be avoided with special LCM caps with outer rails. Microsecond heating of tissue does occur during dissection, but does not appear significant. Must dissolve material off cap.	Newest instrument of group, but several publications citing its use. No clear indication that UV-A laser causes significant degradation of dissected tissue components, but this has not been rigorously tested.	Many publications illustrating isolation of various cell components. No clear indication that UV-A laser causes significant degradation of dissected tissue components, but this has not been rigorously tested.
Special notes	Arcturus has developed an automated system for dissection based on same EVA melting principles with same issues and limitations. This device not reviewed here (cost is said to be \$186K).	Can be used to dissect living cells in culture using special culture dishes. Recently improved quality of membrane coated slides, vastly improving quality of visualization and laser cutting. Unclear if ablation by laser produces micro-aerosol that should be isolated from user.	Can be used to dissect living cells in special culture dishes. Can dissect tissues covered with light sheen of mineral oil allowing better visualization. Unclear if laser pressure catapulting causes microaerosol of tissue particles, and possible respiratory exposure, although this appears possible.
Cost of instrument	Approx \$124K with fluorescence.	Approx \$90K base, approx \$120K with fluorescence and database software	Approx \$160 K

(continued)

Table 1 (Continued)

	Arcturus PixCell IIe	Leica AS LMD	PALM Micro Beam
Cost of disposables	\$3 or more per cap depending on cap type used	Membrane-coated slides required, and cost in range of \$3–4 per slide	No disposables required, but membrane coated slides and coverslips cost in range of \$4 each from PALM, but can be purchased elsewhere
Bottom line: pros	Relatively simple to operate, good track record when: (a) visualization is not a problem, (b) area of interest is not too small, and (c) tissue “wants” to come off slide but does not come off too easily Software is good, easy to use. Company has excellent service record, excellent record of reagent manufacture.	Middle of the road of the three devices in terms of device complexity Controls of focus, $x/y/z$, objective, and illumination convenient to user Excellent software interface Ability to store data in openly accessible database useful for some users. Company has partnered with Qiagen to create reagent systems.	Dissections nearly always work—you are consistently able to get material off the slide. Imaging sufficient for nearly any dissection can be obtained, by selecting from range of objectives, oil/no oil, “liquid cover slip.” Dissector has a large number of useful options in dissecting: combining ablation, laser catapulting, using membrane or not using membrane-coated slides. Excellent software interface, excellent documentation of dissection

Bottom line: cons	Reasonably frequent problems getting targeted tissue to come off slide, wasting tissue and time	All dissections must occur on membrane, cannot use archival slides. Most labs opt to buy membrane slides, whose cost mounts quickly.	More expensive More complex machine, learning curve higher, laser will require more maintenance.
Difficult to get single cells when strong intercellular bonds present or tissue strongly adherent to slide	Dissected regions sometimes do not fall easily into cap. Need to “chase” membrane piece around.	Larger footprint than other two devices	
Magnification not sufficient to do some single cell work. No X100 option available.	In dry environment, static electricity can interfere with gravity-based transfer to tube.	No doubt that many investigators report good results obtaining various biomolecule types from dissections, but rigorous testing of laser pressure catapulting’s effects on nucleic acids and proteins has not been done to our knowledge.	
Visualization often not good, and few parameters to play with because “liquid cover slip” cannot be used	Track record relatively short relative to other two systems, but substantial installed base now present.	User must change objectives manually—can get out of sync with software.	
Special precautions/time needed to reduce potential for contamination by unwanted cells	Proprietary caps cost add up.		

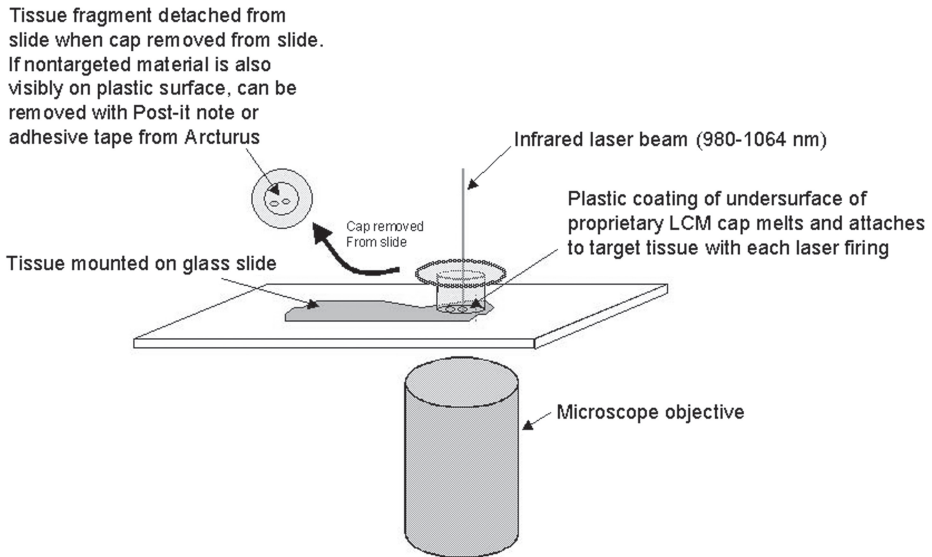


Fig. 2. Arcturus laser capture microdissection (LCM) system.

risk of transfer of nontargeted cells. Viewing the tissue section under the microscope (directly or on a monitor), the investigator activates the infrared laser when the desired ROI is aligned with a targeting laser beam. The infrared laser (980–1064 nm) pulse causes localized melting of the thermoplastic membrane, and expansion of the molten plastic causes it to contact the tissue adjacent to ROI. The EVA plastic then resolidifies, remaining adherent to the targeted ROI. As a result, areas where the EVA has come into contact with targeted tissue become optically clearer, allowing the user to identify easily tissues that have already been targeted. Other descriptions of the LCM process are contained in Eltoun et al. (1), and Curran (25), and others.

A large number of experiments utilizing LCM have been published, illustrating that sufficient DNA, RNA, and protein can be obtained using LCM (24,26–30) for many types of experiments. Momentary heating of the tissue occurs with each contact by the molten EVA, but the effect of this heating generally appears to be minimal based on the success investigators have had with the device to date. Arcturus' PixCell II software allows for visualization and documentation of images of ROI before and after dissection and the tissue transferred to the cap. After the LCM session is complete, the cap is removed from the slide. If the adherence of the EVA plastic to the targeted tissue is stronger than its adherence to the slide, the targeted tissue remains attached to the cap, leaving unwanted tissue regions on the slide, as illustrated in **Fig. 2**.

When dissection is complete for a given cap, Capsure[®] Macro caps with attached microdissected cells are placed onto microcentrifuge tubes containing an appropriate buffer for molecular analysis (caps were engineered to fit Eppendorf brand tubes). Capsure[®] HS caps must be attached to matching ExtracSure[™] extraction devices, which allow extraction in small buffer volumes. The tube is then inverted and incubated as needed to allow microdissected cell components to go into solution.

Positive aspects of the Arcturus LCM technology include:

- Single cells and large areas can be dissected in many situations.
- Single cells can be “cherry-picked” from cell preparation slides.
- Equipment is relatively simple to manage and maintain.
- The laser is solid state and should rarely if ever need replacing.
- The Arcturus company provides generally excellent support including protocols and kits tailored to the LCM system.

Challenges associated with the LCM technology include:

- Transfer of tissue from slide to LCM cap sometimes requires troubleshooting. Tissue that is targeted may remain on the slide after the CapSure[®] cap is removed, indicating that adhesion between the thermoplastic and the tissue is too weak, or the adhesion between the tissue and slide is too strong. Insufficient adhesion of the thermoplastic to the tissue can be caused by insufficient dehydration of the target tissue, which can sometimes be overcome by repeat immersion of the target slide in fresh xylene. When adhesion of the tissue to the glass slide is greater than tissue adhesion to the thermoplastic film, this can be difficult to overcome. Unevenness of tissue surfaces, caused by wrinkled sections or resulting from marked differences in tissue firmness (such as with plant tissues where stiff cellulose is next to soft internal cellular elements, or with tissues containing bone or cartilage), also can make dissection difficult. In some instances, internal bonding within tissue elements prevents selective tearing of individual cells from adjacent cells, preventing LCM altogether.
- It can be costly because of the need to use proprietary Arcturus CapSure[®] caps, which cost in the range of \$2–3 apiece.
- Minimal dissectable area is variable. Single cells can easily be dissected in some tissues, while in others single-cell dissection is impossible because of excessive adhesion to the slide, or because of the above-mentioned resistance to tearing (internal bonding) of target tissue.
- Visualization in some tissues is not adequate. This is most often due to an index of refraction mismatch between the tissue section and air between the tissue and CapSure[™] cap. With the PALM Microbeam and Leica LMD systems, with tissue mounted on membrane-coated slides, a “liquid cover slip” can be placed on the tissue section to provide visualization similar to ordinary cover-slipped sections, without interfering with dissection. This is not feasible with the Arcturus IIe LCM system, as liquid would interfere with adherence between the EVA plastic and the tissue.

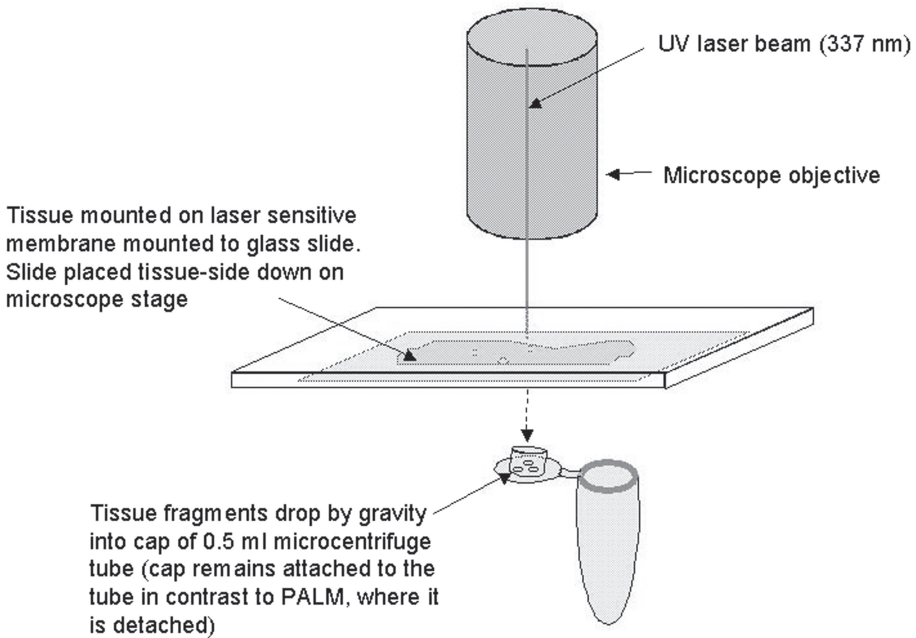


Fig. 3. Leica AS LMD laser microdissection system.

Arcturus recently marketed a new device called Autopix™, which is an automated, enclosed version of the technology contained in the PixCell Iie. Arcturus continues to market the PixCell Iie, which at the time of this writing is probably the most prevalent laser-based microdissection technology. Arcturus uses Olympus microscope in its systems, but has its own service and support team covering all aspects of its devices.

1.6.2. PALM Micro Beam Research System

PALM Microlaser Technologies AG (Bernried, Germany), founded in 1993, originally focused on use of microscope-based ultraviolet (UV) lasers in assisted-reproduction technology such as zona pellucida drilling, but moved into the area of tissue section microdissection when prodded by pathologists and researchers faced with difficulty in obtaining pure cell samples. PALM marketed its first system specifically for tissue microdissection around 1994, and its current device is called the Micro Beam microdissection system. PALM's tissue section microdissection systems differ in a number of important respects from the Arcturus and Leica technologies, and these differences are illustrated in **Figs. 2–4**. The most important positives include the ability to obtain improved visualization by using a “liquid cover slip,” relative predictability of obtaining targeted ROIs, relative predictability in obtaining ROI in the 2- to 5 μm diameter range, rela-

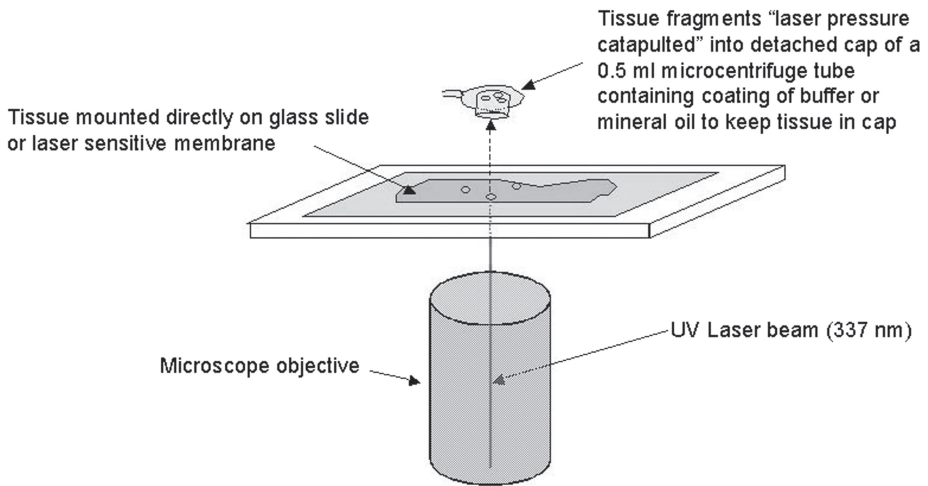


Fig. 4. Zeiss/PALM Micro Beam laser microdissection system.

tive precision of the ablating UV beam (compared to Leica LMD), availability of PALM's patented Laser Pressure Catapulting (LPC) technology for removal of targeted tissue from the slide (as compared to gravity used by Leica), and flexibility in the choice of mounting platforms (glass or special plastic membranes). In addition, the PALM system software was intuitive and adequately sophisticated both for dissections and documentation in our testing. The most important potential challenges with the current PALM technology are as follows: their technology is somewhat more expensive; UV lasers require more maintenance and are more expensive; and in the United States at least, PALM relies on Zeiss for service and support, an arrangement that may be difficult but for which we have no direct experience. PALM recently created a similar arrangement with Olympus in Europe, and it is unclear whether the PALM technology will continue to be supported on both Zeiss and Olympus platforms, and how these platforms might differ. Similar to the Arcturus system, the best current evidence of its effectiveness for a range of molecular studies is the large number of publications using material isolated using the PALM system (31–34).

1.6.3. Leica LMD Microdissection System

Leica was the third major entrant into the laser microdissection device field, and its current Leica LMD system has features that in some ways are hybrids between the Arcturus and PALM systems, as summarized in **Table 1** and **Figs. 2–4**. Similar to the PALM system, its laser is an ablating 337-nm laser in the UV-A range. Similar to the Arcturus, its hardware is compact and its software

is relatively bare bones compared to the PALM software (the version tested in 2003 did not provide more advanced documentation tools, such as allowing direct annotation of images within the Leica software). Positive attributes include several convenient features including a single manual control box for all major dissection functions. These include integration of objective selection and software adjustment to a new magnification (not supported in the PALM version tested), convenient ability to send dissected ROIs from different regions of a slide to separately identified microcentrifuge tubes, and the ability to use ordinary intact microcentrifuge tubes for collection of dissected materials (not possible on either the Arcturus or PALM systems tested). Significant challenges with the Leica system include required use of membrane coated slides, occasional failure of the targeted ROI to drop into the collection tube after dissection, relative lack of fine focus of the UV laser beam compared to the PALM system, and poor performance when using UV illumination. The Leica LMD system is relatively new, but publications citing its use in molecular studies have already appeared (35,36).

Used carefully, all three of the laser-based tissue microdissection systems discussed provide technology sufficient for isolation of most ROI, obtaining material of sufficient quality for many if not most downstream molecular analyses. Selection of the best system for an application should be based on one's specific needs and resources available, taking into consideration the various positive attributes and challenges listed. Technologies are evolving rapidly, so consulting with company websites and representatives is recommended prior to making purchase decision. More recently marketed tissue microdissection systems from Bio-Rad (Hercules, CA) and MMI AG (Glottbrugg, Switzerland), and other new entrants have not been reviewed by us but should also be considered.

2. Materials

2.1. Rapid Tissue Freezing

2.1.1. Isopentane Method

1. Isopentane.
2. Dry ice.
3. Ice bucket.
4. Metal bowl, 500-mL or larger volume.
5. Metal or Plexiglas basket corresponding to metal bowl to hold tissue while freezing.

2.1.2. Gentle-Jane® Method

1. Gentle-Jane® (Instrumedics) snap-freezing device.
2. Liquid nitrogen.
3. OCT ("optimal cutting temperature") compound (Tissue-Tek, others), Cryogel (Instrumedics), or other embedding medium.

2.2. Preparation of Cytologic Specimens for Microdissection or Direct Molecular Analysis

2.2.1. Tissue Disaggregation Methods

Solid tissues can be disaggregated to obtain pure cell mixtures ready for further purification and/or live cell experiments or for direct molecular separation and phenotyping.

2.2.1.2. ENZYMATIC AND/OR MECHANICAL DISAGGREGATION

1. Fresh tissue to be disaggregated. (This methods may also work on frozen tissues [37], and success may be increased if the tissues are frozen in media containing dimethyl sulfoxide (DMSO), that may protect cell membranes.)
2. Sterile solution compatible with cells of interest such as RPMI-1640 or Hank's balanced salt solution (HBSS), with addition of serum, defined additives (growth factors, insulin, cytokines, etc.), and antibiotics as desired.
3. Enzyme(s) such as collagenase 1A (Sigma, St. Louis, MO, USA) to be used for disaggregation (see example) if needed.
4. Sterile scalpels and/or automated mechanical disaggregation system (such as Medi-machine™ marketed by BD (formerly Becton Dickenson, Franklin Lakes, NJ).

2.2.2. Direct Smears of Liquids Containing Suspended Cells

1. Standard (uncoated) glass microscope slides.
2. 50–250 μ L of fluid to be smeared.
3. Hemocytometer cover slip or other device narrower than glass slide to use as a cell spreader.
4. 70% ethanol.

2.2.3. Tissue Scraping

Fresh tissues containing cells that can be detached intact with mild shearing force can be rapidly sampled by scraping with a sterile scalpel blade and then rapidly spreading the scraped liquid sample onto a glass slide with the blade. A basic requirement for use of this technique together with microdissection is that the intended target cells can be readily identified cytologically or by cytology together with specific staining characteristics.

2.2.4. Cell Concentration Technologies

2.2.4.1. DENSITY AND SIZE GRADIENT SEPARATIONS

Many types of cells in solution can be separated by density and/or size, without significant damage. For example, from disaggregated living liver cells, hepatocytes, Kupffer cells, liver endothelial cells, and bile duct epithelial cells can be separated using density gradients (38):

1. Percoll (Amersham), RediGrad (Amersham), Ficoll-Paque Plus (Amersham), others.
2. Sterile 50-mL conical tubes.
3. Diluent (usually phosphate-buffered saline or similar).
4. Red cell lysis buffer (ACK by Bio-Whittaker, others).
5. Centrifuge.

2.2.4.2. MAGNETIC-BEAD OR OTHER BEAD-BASED SEPARATIONS

Immunobead-based separation has been used effectively for concentrating multiple myeloma cells from fresh bone marrow aspirates (39) for molecular analysis. With a well-optimized combination of disaggregation methods and specific antibodies, other types of fresh or frozen tissue can be separated using a combination of density gradient and magnetic-bead separations. Methods will vary depending on tissues separated and target cells desired. Listed here is a general pattern.

1. Aqueous sample containing intact cells of interest admixed with other cells.
2. Erythrocyte lysis buffer (if needed). Two such buffers are: 0.32 *M* sucrose, 10 mM Tris-HCl, 5 mM MgCl₂, 1% Triton X-100, or alternatively hypotonic solutions (0.15 *M* NH₄Cl in 0.1 mM Tris-HCl).
3. Appropriate nonspecific (blocking) and specific antibodies (multiple suppliers) for selection of wanted or unwanted cells.
4. Paramagnetic beads coated with secondary antibody specific to the primary antibodies used (Dynal, Oslo, Norway, and others).
5. Magnet (Dynal, others) for separation of beads, leaving target cells in supernatant, or on beads as designed.

2.2.4.3. SEMI-AUTOMATED CELL CONCENTRATION METHODS

These include Cytospin® (Thermo Shandon Inc.) preparations, or by more advanced thin-layer technology called ThinPrep® (Cytoc Corp.) or AutocytePrep (TriPath Imaging). The Cytospin® technique uses proprietary centrifuge cartridges to separate cells in suspension and place them on specific areas of a microscope slide ready for staining. ThinPrep® also uses proprietary solutions (separate protocols for fine-needle aspirates, body fluids, and mucoid samples) and instrumentation to produce cells on slides. Tripath's solution is targeted at Papanicolaou cervical smears but could be modified for other purposes. Consult the manufacturer's manual for details.

2.3. Chemical Fixatives

Alcohol-based and formaldehyde-based tissue fixation methods are presented, as they currently come closest to providing both sufficient histologic detail for light microscopy and sufficiently preserved material for molecular analysis of varying types. Metal-based fixatives (Zenker's, Harris hematoxylin, etc.) can

provide excellent histology but can interfere with many molecular analyses and are not discussed.

2.3.1. 10% Neutral Buffered Formalin (10% NBF)

(see **Notes 1 and 2**)

Many laboratories purchase 10% NBF ready-made from a wide variety of suppliers. It is relatively easy to make, however, and in some instances (see **Notes**) making your own may provide significant advantages:

1. Fume hood.
2. 2-L graduated cylinder with stir bar.
3. Magnetic stirring plate.
4. 1 L of 10% formalin (100 mL of 37% formaldehyde diluted 1:10).
5. 4 g of sodium phosphate monobasic ($\text{Na}_2\text{H}_2\text{PO}_4 \cdot \text{H}_2\text{O}$).
6. 6.5 g of sodium phosphate dibasic (anhydrous) ($\text{Na}_2 \text{H}_2 \text{PO}_4$).

In a fume hood, add stir bar, sodium phosphate monobasic, and sodium phosphate dibasic to empty graduated cylinder on stir plate (see **Note 3**). Add 900 mL of water followed by 100 mL of 37% formaldehyde (see **Note 4**). Stir until phosphate dissolves (see **Note 5**).

2.3.2. Alcohol-Based Solutions (see **Note 6**)

1. 70% ethanol (see **Note 7**). To make 1 L, add 700 mL of 100% ethanol to a graduated cylinder, and add water to 1 L. The amount of water needed to reach 1000 mL will be roughly 330 mL because of volume contraction caused by hydrogen bond formation.
2. 4.2:2.0:1.8 (v/v/v) Ethanol–methanol–water fixative (see **Notes 8–10**). To make 8 L, to a 10-L graduated cylinder, add 1800 mL of H_2O , 2000 mL of 100% methanol, and 4200 mL of 100% ethanol. Mix well with a stir bar and bring the volume up to 8 L with water) to make up for volume shrinkage due to hydrogen bonding.

2.4. Combined Formalin Fixation/Sucrose Infusion for Cryostat Sectioning

In situations in which standard frozen sections may not provide adequate histology for downstream microdissection, and where standard formalin fixation and paraffin embedding will not provide adequate DNA, RNA, or protein quality, an alternate approach is to perform short-term fixation with freshly prepared 2% or 4% buffered formaldehyde (as opposed to using commercially prepared 10% formalin, which is 4% formaldehyde but also contains a small amount of methanol, which coagulates proteins), then rinsing the sample with glycine (to remove excess formaldehyde and inactivate formaldehyde) followed by sucrose infusion.

1. Fresh 2% or 4% neutral buffered formaldehyde, or modified Millonig formalin (see **Subheading 4.3.1.**) if isoosmotic fixation is desired. Note that this freshly made “formaldehyde” is the same as what many call “paraformaldehyde.”
2. Sterile phosphate-buffered saline (PBS).
3. 0.5 M Sucrose in PBS.

2.5. Standard Automated Clearing and Paraffin Embedding of Fixed Tissues

Most molecular laboratories will work with histology laboratories for tissue clearing (usually with xylenes) and embedding (usually with paraffins). Modern paraffin tissue embedding occurs almost entirely in automated processors under vacuum. Molecular researchers must recognize, however, that standard surgical pathology tissue processors may start with a formalin step, and thus if formalin exposure is to be avoided, a separate no-formalin processor run must be created. In addition, processors on which formalin is routinely used may allow formalin exposure even when not selected. Higher temperatures and longer exposure times than desired may also be commonplace. Examination of these parameters with one's support laboratory is recommended.

Paraffins for embedding vary in melting temperature and quantity of added polymer plastic as stiffening agent. Increased polymer allows greater stiffness and thinner sectioning, but also increases infiltration time.

2.6. Sectioning of Tissue Blocks for Microdissection

2.6.1. Frozen Sections

1. Cryostat.
2. OCT or other embedding compound.
3. Mounting chucks for cryostat.
4. Dry ice in styrofoam container.

2.6.2. Paraffin Sections

1. Microtome.
2. Water bath filled with deionized/distilled ddH₂O for floating sections.
3. Clean, disposable blade.
4. Oven for baking slides.

2.7. Staining of Tissue Sections for Microdissection

2.7.1. Mayer's Hemalum and Eosin Staining for DNA, RNA, and Protein Recovery

1. Fresh xylenes (Sigma, others).
2. Fresh 100%, 95%, 70% ethanol (use diethyl pyrocarbonate [DEPC]-treated water for dilution of 100% ethanol).

3. Mayer's hematoxylin (Sigma, Richard-Allan, others).
4. Eosin Y (Sigma, Richard-Allan, others).
5. Mini-protease inhibitor tablets (Roche) (if protein recovery is desired).

2.7.2. Immunostaining Prior to Laser Microdissection

1. DEPC-treated H₂O (Invitrogen/Research Genetics) (for preparation of PBS, alcohols).
2. Superfrost Plus glass slides (Fisher Scientific).
3. Cold acetone.
4. 1X PBS, pH 7.4.
5. DAKO Quick Staining kit (DAKO Corp.), a three-step streptavidin–biotin technique with prediluted mono- or polyclonal (rabbit) primary antibodies optimized for very short staining times.
6. Diaminobenzidine (DAB).
7. Hematoxylin solution, Mayer's (Sigma).
8. 70%, 95%, 100% ethanol.
9. Xylenes, mixed, ACS grade (Sigma).
10. Placental RNase inhibitor (Perkin Elmer, Branchburg).

2.7.3. Methylene Blue Staining

1. Methylene blue (0.05% in water, Sigma 31911-2).
2. DEPC-treated water (Invitrogen/Research Genetics, others).

2.7.4. Methyl Green Staining

1. Methyl green solution (actually "ethyl green;" see **Subheading 1.3.**) (Dako, S1962).
2. DEPC-treated water (Invitrogen/Research Genetics, others).

2.7.5. Nuclear Fast Red

1. Nuclear fast red solution (Dako, S1963).
2. DEPC-treated water (Invitrogen/Research Genetics, others).

2.8. Manual Microdissection

2.8.1. Cryostat-Based Microdissection of Tissue Blocks

1. Cryostat with blade guards.
2. OCT or other embedding compound.
3. Brightfield microscope.
4. Glass slides.
5. 50-mL conical tubes.
6. 16-in. long smooth forceps.
7. Insulated bucket containing liquid nitrogen.
8. Sterile one-sided straight edge blades.
9. Mayer's hemalum and eosin slide staining setup.
10. Logbook or computerized logging system.

2.8.2. Manual Microdissection of Tissue Sections on Glass Slides

The method described is a combination of the one in use in the NCI Pathogenetics Unit and the one described by Moskaluk and Kern (40):

1. Stained glass-slide-mounted sections without coverslips and adjacent stained, cover-slipped sections.
2. Dissecting microscope or standard inverted microscope with hydraulic micromanipulator arm, if available.
3. Sterile 30-gage needle on syringe.
4. 2.5% Glycerol solution.
5. Agarose and buffers as needed for specific dissections.

2.9. Laser Microdissection of Tissue Sections on Slides

2.9.1. Arcturus LCM System

1. LCM device.
2. CapSure dissection caps.
3. Post-It® Notes or Arcturus PrepStrips™.
4. Perkin Elmer/Applied Biosystems GeneAmp 500-μL thin-walled polymerase chain reaction (PCR) reaction tubes, cat. no. N8010611.

2.9.2. PALM Microbeam System

2.9.3. Leica LMD System

2.10. Isolation of Analyte from Microdissected Materials

2.10.1. Isolation of DNA

DNA isolation kits are available from a number of manufacturers. For small samples, kits may be more cost-effective than nonkit methods. For large samples (if >10 μg of DNA will be isolated), we believe nonkit methods are most cost effective.

2.10.1.1. KIT-BASED METHODS

A number of kits are available for DNA isolation. For LCM-derived materials, we tested Trizol, DNAzol, Trireagent, Easy-DNA (Invitrogen), and the DNeasy kit (Qiagen), against sodium dodecyl sulfate (SDS)–phenol–chloroform extraction, and Tween-20–phenol–chloroform extraction, and of the kits, the DNeasy kit performed best in our hands (GSB Laboratory), although SDS–phenol–chloroform provided a better yield, in our hands, DNA size is generally larger with DNeasy than with phenol–chloroform extraction. DNeasy is also easier to use because of its lower toxic chemical content. We describe a method using the DNeasy kit and LCM caps, although this could be applied to microdissected material from any source. Several more recently marketed DNA isolation kits are also available, but have not been tested by us.

1. DNeasy kit (Qiagen) or similar kit. The DNeasy kit contains 2-mL collection tubes, a series of proprietary ("black box") buffers: ATL (lysis buffer), AL (lysis buffer contains guanidine hydrochloride), AW1 wash buffer (contains guanidine hydrochloride), AW2 wash buffer, AE elution buffer; also proteinase K (activity = 600 mAU/mL solution or 40 mAU/mg of protein).
2. LCM cap removal device (if LCM caps are source of DNA).
3. 100% (Absolute) ethanol.

2.10.1.2. SDS/PHENOL-CHLOROFORM EXTRACTION

We compared Tween-20-phenol-chloroform extraction to SDS-phenol-chloroform extraction, and in our hands (GSB laboratory), SDS-phenol-chloroform extraction provided double or greater yields, and better quality DNA. Our SDS-phenol-chloroform extraction method is described here and is based on the method of Goelz et al. (41). The procedure given is for large quantities of DNA (10–1000 μ g), and can be scaled down for smaller samples.

1. 1 L of DNA digestion buffer.
 - a. Sterile bottle.
 - b. 750 mL of sterile, deionized/distilled H₂O (ddH₂O).
 - c. 50 mL of 1 M Tris-HCl (pH 8).
 - d. 100 mL of 0.5 M EDTA, pH 8.
 - e. 100 mL of 20% SDS.Combine the ingredients in the bottle. Gently add 20% SDS to avoid foaming. Gently mix the solution, and label the bottle: DNA extraction buffer: 50 mM Tris-HCl, 50 mM EDTA, 2% SDS. This can be stored at room temperature. Place 6 mL in a sterile 50-mL conical tube to isolate DNA from 100 to 300 6- μ m frozen tissue sections. Scale down for smaller amounts of tissue.
2. DNA digestion materials.
 - a. 5 mL of proteinase K (15.6 mg/mL) (Roche Molecular Biochemicals, cat. no. 1-373-196).
 - b. Incubator.
 - c. Rocking device, such as Belly-Button Shaker/Rocker (Stovall Life Science, Inc.). Set the incubator (or water bath) to 48°C and allow to equilibrate. To each sample (6 mL), add 38 μ L of proteinase K. Incubate on undulating Belly Button (Stovall) or other gentle agitation device at 48°C for 12–18 h (typically overnight) (*see* **Notes 11** and **12**).
3. 50 mL of LoTE DNA suspension buffer.
 - a. Sterile 50-mL conical tube.
 - b. 44.5 mL of ddH₂O.
 - c. 450 μ L of 1 M Tris-HCl, pH 8.0.
 - d. 9 μ L of 0.5 M EDTA, pH 8.0.Add ddH₂O, Tris-HCl, and EDTA to a conical tube; mix well. Filter sterilize if desired. Label tube. Final concentrations are: 10 mM Tris-HCl, 1 mM EDTA. Keep refrigerated.

4. DNA purification.

- a. One box of serum separation tubes (SST) (Becton Dickinson-Vacutainer Systems, cat. no. 366512). (Also consider using Phase Lock Gel™ tubes of various sizes, marketed by Eppendorf.)
- b. Phenol–chloroform–isoamyl alcohol (25:24:1 v/v/v) (UltraPure Gibco BRL).
- c. Sterile 50-mL conical tubes.
- d. Centrifuge and rotor capable of handling SST and 50-mL conical tubes.

2.10.2. Isolation of RNA

2.10.2.1. RNASE AWAY™ (MOLECULAR BIOPRODUCTS) OR OTHER RNASE INACTIVATING SOLUTION

2.10.2.2. KIT-BASED METHOD (PICOPURE™ RNA ISOLATION KIT BY ARCTURUS)

This kit contains several proprietary (“black box”) buffers, including conditioning buffer (CB), GITC-based extraction buffer (XB), 70% ethanol (EtOH), wash buffer 1 (W1), wash buffer 2 (W2), elution buffer (EB), RNA purification columns, collection tubes, and microcentrifuge tubes. Although the description is focused on material derived from LCM, it is equally applicable to tissue isolated using any type of microdissection. Small-quantity RNA isolation kits are also available from Ambion, Qiagen, and others.

2.10.2.3. PHENOL–CHLOROFORM BASED RNA EXTRACTION METHOD

1. Rnase-free microcentrifuge tubes.
2. DEPC-treated water purchased ready for use (Invitrogen, Sigma, others) or made by adding 0.2 mL of DEPC (Sigma and others) per 100 mL of ddH₂O, shaking vigorously to get DEPC into solution, and autoclaving to inactivate remaining DEPC. **Caution:** Handle DEPC only in a fume hood, as it may be a carcinogen.
3. 30 mL of GITC denaturing solution made with 29.3 mL of ddH₂O, 1.76 mL of 0.75 M Na citrate, pH 7.0, 2.64 mL of 10% (w/v) *N*-lauroylsarcosine, 25.0 g of guanidine thiocyanate (dissolves with stirring at 65°C), and 35 µL of 2-mercaptoethanol. Final concentrations are 4 M guanidinium isothiocyanate, 0.5% *N*-lauroylsarcosine, 25 mM sodium citrate, and 0.1 M 2-mercaptoethanol.
4. 2 M Sodium acetate pH 4 (add 1.64 g of anhydrous sodium acetate to 4 mL of water and 3.5 mL of glacial acetic acid, and bring the volume to 10 mL with ddH₂O).
5. Water-saturated buffered phenol. Dissolve 10 g of phenol crystals in ddH₂O at 65°C. Mix dissolved phenol with 200 mM Tris-base until the aqueous solution reaches pH 8. Remove the upper water phase and store at 4°C for up to 1 mo.
6. 70% Ethanol (prepared with DEPC-treated water).
7. 49:1 (v/v) chloroform–isoamyl alcohol.
8. 100% isopropanol.
9. Refrigerated microcentrifuge or other refrigerated centrifuge capable of handling microcentrifuge tubes.
10. Tissue disaggregating device, as needed.

11. Formamide purchased ready for use (Sigma, Molecular Research Center, others) or prepare in the laboratory by mixing with 1 g of AG 501-X8 ion-exchange resin (Bio-Rad, others) per 10 mL of formamide for 45 min and filter at room temperature.

2.10.2.4. DNase TREATMENT OF TOTAL RNA

1. DEPC-treated ddH₂O (*see Subheading 2.10.2.3.* for recipe).
2. RNase inhibitor (Perkin Elmer).
3. DNase I (GenHunter Inc) and DNase I buffer (GenHunter, Inc.).

2.10.3. Isolation of Protein for 2-Dimensional Gel Electrophoresis

Protein extraction methods vary widely depending on the intended analysis method and are not detailed here. Recent papers by Ornstein (9), Simone (10), and Emmert-Buck et al. (42) contain useful protocols for protein extraction from microdissected frozen tissue material, and a 1998 paper by Ikeda et al. (43) suggests that paraffin-embedded tissue can also be used for proteomic analysis.

3. Methods

3.1. Rapid Tissue Freezing (*see Notes 13 and 14*)

3.1.1. Isopentane Method

1. Place 400 mL of isopentane (highly flammable) in metal container large enough to hold a corresponding Plexiglas or metal basket (*see Note 15*).
2. Place bed of dry ice in ice bucket.
3. Place the metal container containing isopentane onto dry ice. Allow 15–30 min for a cool-down period. Put the tissue to be frozen into the metal or Plexiglas basket long enough for complete freezing, Excess isopentane (highly flammable) can be removed with a paper towel (*see Note 16*).

3.1.2. Gentle-Jane® Method

In our hands, because of the rapidity of freezing, this method provides superior frozen section histology, and in some cases provides as good as or better histology than from formalin-fixed, paraffin-embedded samples (*see Note 17*). Rapid freezing reduces ice crystal size, preserving cellular architecture. Significant drawbacks are covered in the **Notes** (*see Note 18*).

1. Place sufficient liquid nitrogen in the Gentle-Jane (Instrumedics) liquid nitrogen container to cover the metal piston (*see Note 19*).
2. Wait until liquid nitrogen stops boiling (metal and liquid nitrogen at equal temperatures).
3. Place tissue on freezing pedestal.
4. Move metal piston to piston-holder, and gently lower onto tissue located on the freezing pedestal.
5. Remove the frozen tissue from the pedestal.

3.2. Preparation of Cytologic Specimens for Microdissection

3.2.1. Tissue Disaggregation Methods

A generic method for tissue disaggregation is defined below. Specific methods will need to be optimized depending on the specific input tissues and output needs of the method. The method described is based on reports by Novelli et al. (44) of the use of collagenase and the Medimachine™ for isolation and downstream analysis of cutaneous lymphocytes (44) and by Brockhoff et al. for isolation of colon carcinoma cells for downstream flow cytometry (37).

3.2.1.1. ENZYMATIC AND/OR MECHANICAL DISAGGREGATION (SEE NOTE 20)

1. Mechanically disaggregate tissue using sterile scalpel blades or the Medimachine™ as recommended by the manufacturer (http://www.bdbiosciences.com/immunocytometry_systems/brochures/pdf/mmach_download.pdf). The Medimachine system requires selection of the cutting device (Filcon™) mesh pore size desired (35 or 50 μm ; 30 μm is for isolation of nuclei, 50 μm for whole cells), and the specific filter pore shape (syringe type for larger volumes, cup type for smaller volumes) and pore size desired (10–500 μm). Alternatively, if manual mechanical disaggregation is performed with sterile scalpel blades, 70- μm (or other) pore size tissue strainers (Falcon, BD Inc., Franklin Lakes, NJ) can be used.
2. If desired, either prior to (if the tissue sample is very small) or after mechanical disaggregation, the suspended tissue clumps can be exposed to disaggregating enzymes such as collagenase 1A (Sigma). Overexposure can lead to decreased cell integrity and viability, so optimization of exposure duration and temperature is critical for success.

3.2.2. Direct Smears of Liquids Containing Suspended Cells (see NOTE 21)

1. Place one drop of liquid sample on a glass slide.
2. Drag the hemocytometer cover slip over the liquid sample.
3. Drop immediately (before drying) into fresh 70% ethanol for 10 dips. Then air-dry or proceed to staining.

3.2.3. Tissue Scraping (see NOTES 22 and 23)

1. Gently scrape a sterile blade against wet tissue surface to collect fluid on the edge of the blade.
2. Drag the blade gently across the dry glass slide.
3. Immediately plunge into 70% ethanol for 20 dips, then proceed to staining or air-dry.

3.2.4. Cell Concentration Technologies

3.2.4.1. DENSITY AND SIZE GRADIENT SEPARATIONS (SEE NOTE 24)

1. Dilute cells, and prepare gradient in 50-mL conical tubes as directed by the manufacturer.

2. Centrifuge the cells as directed by manufacturer (usually approx 10,000g if cells are in saline), wash if desired, and recentrifuge.
3. Lyse red cells if desired, wash, recentrifuge, resuspend, and count cells before downstream molecular analysis or further separation by other methods.

3.2.4.2. MAGNETIC-BEAD BASED SEPARATIONS (SEE NOTE 25)

Magnetic-bead (or other bead-based) separations can be used separately or together with microdissection-based isolation. For particularly bloody specimens, red blood cell lysis agents should be added to bead-based protocols. Density-based separation methods (such as Ficoll gradients) can also add power to bead-based techniques.

1. Obtain sterile aqueous samples containing intact cells of interest admixed with other cells.
2. If necessary, use density gradient techniques to remove erythrocytes or use erythrocyte lysis buffer such as those listed.
3. Wash cells and incubate with appropriate nonspecific (blocking) and specific antibodies for selection of wanted or unwanted cells.
4. Wash cells and incubate with paramagnetic beads coated with secondary antibody directed against the primary antibodies used (Dynal, Oslo, Norway, and others).
5. Magnetic (Dynal, others) separation of beads, leaving target cells in supernatant, or on beads as desired.

3.2.4.3. SEMI-AUTOMATED CELL CONCENTRATION METHODS (SEE NOTE 26).

For Cytospin® (Thermo Shandon, Inc.), ThinPrep® (Cytoc Corp.), or Auto-cytePrep (TriPath Imaging) contact the manufacturer for specific materials and methods.

3.3. Combined Formalin Fixation/Sucrose Infusion for Cryostat Sectioning (see Note 27)

1. Fix samples (dimensions as small as possible to allow rapid penetration of fixative) in 2% or 4% buffered formaldehyde at 4°C for 4–24 h (duration needs to be optimized for your tissue/application). When possible, perfuse the entire organ with saline followed by formaldehyde prior to sectioning and placement in fixative solution. Usually this can be accomplished only via volume replacement through the left ventricle.
2. Wash samples with sterile 4°C PBS.
3. Place samples in 0.5 M sucrose in PBS at 4°C. When tissue sinks, infusion is complete. Do not allow the infusion to go longer than 1 d (reduces quality of tissue morphology).
4. Place tissue samples in OCT or other embedding compound, and freeze rapidly. Section on a cryostat.

3.4. Standard Automated Clearing and Paraffin Embedding of Fixed Tissues (see Note 31)

See **Subheading 2.5.** for comments. In terms of standard embedding materials, we have had good results with type VI paraffin (Richard Allan Scientific, Kalamazoo, MI), but many other products are available that are suitable.

3.5. Sectioning of Tissue Blocks for Microdissection

3.5.1. Frozen Sections (see Notes 29 and 30)

- 1. Place a small amount of OCT onto a chuck in the cryostat.
- 2. Immediately place the frozen tissue block onto the chuck.
- 3. After the block is adherent to the chuck, place it in position onto the microtome.
- 4. Proceed to cut tissue sections onto glass slides. Reference H&E slides are cut at 6- μ m thickness, slides for microdissection are typically cut at 8- μ m, or thicker if ROI is still distinguishable at this thickness.
- 5. Immediately place the slides onto dry ice. Slides can be stained and dissected or stored at -80°C.

3.5.2. Paraffin Sections (see Notes 31 and 32)

- 1. Place tissue block onto a microtome.
- 2. Proceed to cut tissue sections.
- 3. Sections are floated on the surface of a bath containing distilled deionized water (ddH₂O) at 39°C.
- 4. Floating sections are lifted onto glass slides.
- 5. Slides are baked at 60°C for 5–10 min, until the wax surrounding the tissue goes from whitish to translucent.

3.6. Staining of Tissue Sections for Microdissection

3.6.1. Mayer’s Hemalum and Eosin Staining for DNA, RNA, and Protein Recovery (see Notes 33 and 34)

- 1. Replace reagents frequently for optimal results, and to reduce the risk of cross contamination.
- 2. If protein recovery is desired from sections, add one complete miniprotease inhibitor tablet (Roche) per 10 mL of reagent, except xylene.
- 3. Stain each slide-mounted section as follows (*see Notes 35 and 36*):

Reagents in order of treatment	Time
Fresh xylenes 1 (to remove paraffin)	3 min
Fresh xylenes 2 (to remove paraffin)	2 min
100% ethanol	15 s
95% ethanol	15 s
70% ethanol	15 s
DEPC-treated water	10 s

Reagents in order of treatment	Time
Mayer's hematoxylin	6–30 s (optimize to tissue)
DEPC-treated water	10 s
70% ethanol	15 s
Eosin Y	1–5 s (optimize to tissue)
95% ethanol	15 s
95% ethanol	15 s
100% ethanol	15 s
100% ethanol	15 s
Fresh xylenes (for dehydration)	60 s
Air dry (allow xylenes to evaporate)	2 min

Sections are now ready for microdissection.

3.6.2. Immunostaining Prior to Laser Microdissection (see **Notes 37–39**)

Frozen sections are immunostained under RNase-free conditions using a rapid three-step streptavidin–biotin technique followed by dehydration.

1. Cut 8- μ m thick serial sections of snap-frozen tissue blocks on a standard cryostat with a new disposable cryostat blade.
2. Mount the tissue sections on Superfrost Plus glass slides and store immediately at -80°C .
3. Thaw the frozen sections at room temperature for 30–60 s without drying.
4. Fix by immersing immediately in cold acetone for 5 min.
5. Rinse the slides briefly in 1X phosphate-buffered saline (PBS, see recipe in **Notes**), pH 7.4. (see **Note 40**).
6. Using the DAKO Quick Staining kit, immunostain the slides by incubating the slides at room temperature with the primary and avidin-linked secondary antibodies and the horseradish peroxidase for 90–120 s each, rinsing briefly with 1X PBS between each step.
7. Develop the color with diaminobenzidine (DAB) for 3–5 min in the presence of dilute H_2O_2 and counterstain with Mayer's hemalum for 15–30 s.
8. Dehydrate the sections sequentially in 70%, 95%, 100% ethanol (15 s each), and xylenes (twice for 2 min each).
9. Air-dry.
10. The immunostained sections are then ready for LCM.

3.6.3. Methylene Blue Staining (see **Note 41**)

Reagents in order of treatment	Time
Fresh xylenes ¹ (to remove paraffin)	3 min
Fresh xylenes ² (to remove paraffin)	2 min
100% ethanol	15 s
95% ethanol	15 s
70% ethanol	15 s

Reagents in order of treatment	Time
DEPC-treated water	10 s
Methylene blue	5–10 min
DEPC-treated water	10 s
Fresh xylenes (for dehydration if using Arcturus PixCell)	60 s
Air-dry (allow xylenes to evaporate)	2 min

3.6.4. Methyl Green Staining (see **Note 42**)

Reagents in order of treatment	Time
Fresh xylenes 1 (to remove paraffin)	3 min
Fresh xylenes 2 (to remove paraffin)	2 min
100% ethanol	15 s
95% ethanol	15 s
70% ethanol	15 s
DEPC-treated water	10 s
Methyl green solution	5 min
DEPC-treated water	10 s
Fresh xylenes (for dehydration if using Arcturus PixCell)	60 s
Air-dry (allow xylenes to evaporate)	2 min

3.6.5. Nuclear Fast Red (see **Note 43**)

Reagents in order of treatment	Time
Fresh xylenes 1 (to remove paraffin)	3 min
Fresh xylenes 2 (to remove paraffin)	2 min
100% ethanol	15 s
95% ethanol	15 s
70% ethanol	15 s
DEPC-treated water	10 s
Nuclear fast red solution	5–10 min
DEPC-treated water	10 s
Fresh xylenes (for dehydration if using Arcturus PixCell)	60 s
Air-dry (allow xylenes to evaporate)	2 min

3.7. Manual Microdissection

3.7.1. Cryostat-Based Microdissection of Tissue Blocks

The advantages of this cryostat-based dissection technique are that a large amount of material can be obtained for analysis in a relatively short time, with minimal required equipment and expertise needed, and easy to manage documentation. The disadvantage is that even the highest possible purity obtained using this technique is not sufficient for some experiments.

1. Mount the block to be dissected in the cryostat using OCT or other mounting medium. Be sure to surround the block with mounting media so it has a strong base.
2. Cut face section of tissue to be dissected, mount on an ordinary glass slide, stain with H&E, and cover slip.
3. Examine section microscopically with $\times 4$, $\times 10$ objectives and clearly mark unwanted areas with a lab marker. Label slide as section 1 (etc.) and record in the logbook.
4. Remove the cryostat blade, or cover with a knife guard. Lock the reciprocating block holder in place using the cryostat-locking mechanism. Place the marked glass slide against the block to identify area to be cut. Draw dots or lines directly on frozen block with the marker to indicate where cuts should be made.
5. Use new heavy-duty single-edge blade to carefully cut away unwanted tissue, and trim OCT from edge of block, leaving a wider base of OCT close to the cryostat chuck (reduces risk that block will pop off chuck after dissection) **Caution:** Take great care to avoid cutting yourself (*see Note 44*).
6. Cut another face section of the block and mark this slide as section 2. Examine to be sure that all unwanted tissue has been dissected. Repeat dissection and staining until only desired tissue remains. Estimate the percentage purity of dissected material and record in logbook.
7. Cut fifty 6- μm sections serially and allow them to stack on the cryostat cutting plate (which is kept clean). Place collected sections in a cold sterile prelabeled 50-mL conical tube using long forceps prechilled by dipping in liquid nitrogen just prior to collection. Keep everything that contacts the sections frozen to avoid tissue sticking to the forceps and to the sides of the 50-mL conical tube (*see Note 45*).
8. To monitor dissection progress, interval sections are mounted on glass slides and H&E stained (usually before and after each episode of dissection at 300- to 600- μm intervals). In some cases undesired areas of the frozen block are easy to identify grossly based on their appearance in the initial face section, allowing continuous visual monitoring and removal of undesired areas without the need to repeat slide mounting and staining.
9. A record of estimated purity of the resulting sample, the number of sections, and cross-sectional area dissected should be kept for reference during analysis of molecular data and for monitoring of the percentage yield in downstream DNA/RNA/protein isolation protocols (*see Note 46*).

3.7.2. Manual Microdissection of Tissue Sections on Glass Slides

1. Prepare stained sections 7–10 μm thick, on glass slides without cover slips. For each section to be dissected, prepare an adjacent stained cover slipped section on a glass slide as a “scout section.” Prepare a dissection station using a standard inverted microscope using a 30-gage needle on a syringe as the microdissecting tool (*see Note 47*).
2. If desired (test necessity of this step for tissues to be dissected), place sections for dissection in 2.5% glycerol solution.
3. While viewing the tissue through the microscope, gently scrape the cell population of interest with the needle. The dissected cells will become detached from the

slide and form small dark clumps of tissue that can be collected on the needle by electrostatic attraction. Several small tissue fragments can be procured simultaneously. Collection of an initial fragment on the tip of the needle will assist in procuring subsequent tissue.

4. The tip of the needle with the procured tissue fragments should be carefully placed into a 1.5-mL tube containing the appropriate buffer. Gentle shaking of the tube will ensure the tissue detaches from the tip of the needle (*see Notes 48–51*).

3.8. Laser Microdissection of Tissue Sections on Slides

3.8.1. Arcturus LCM System

The described procedure is for use with the Pixcell I or II Laser Capture Microdissection System manufactured by Arcturus Corporation (Santa Clara, CA), and assumes rudimentary knowledge of the function of the components of the instrument and the software that accompanies the instrument. The procedure can be divided into three basic steps: slide positioning, microdissecting with the laser, and collecting the microdissected cells. Additional methods information can be found at the Arcturus Engineering website (www.arctur.com).

3.8.1.1. POSITIONING THE SLIDE FOR MICRODISSECTION

Wear gloves when microdissecting to avoid contamination of LCM specimens. Clean the microscope stage and capping station with 95% ethanol before beginning the microdissection to reduce the possibility of contamination. With the stage vacuum off, place the glass slide with the section to be microdissected on the microscope stage and identify the target of interest. When viewing uncovered sections for dissection, put the light diffuser piece (white membrane) in place to improve visualization. If necessary, refer to an adjacent cover slipped section for orientation. When the target of interest is identified, place the stage-motion joystick so that it is perpendicular to the tabletop and then slide the glass slide so that the target of interest is in the center of the field of view. Turn on the stage vacuum. If the slide does not cover the vacuum chuck holes when centered, move the slide as little as necessary until it just covers the holes and continue to the next step. Identify targets of interest and take “roadmap” images of targets of interest if desired using the Arcturus software.

3.8.1.2. MICRODISSECTION

Pick up a CapSure™ LCM cap from the loaded cassette module with the placement arm, moving the placement arm toward the caps to ensure that the cassette module is engaged so that the first available cap is aligned, and lifting the transport arm until the cap detaches from the base slide in the cassette module. The arm must then be positioned over the tissue, ensuring that the area to be

microdissected is still in the microscopic field of view. Finally, the arm is gently lowered so that the cap contacts the tissue section. If there are folds in the tissue, the cap may not make direct contact with the entire surface at that area and transfer efficiency is compromised. Therefore, it is advisable to inspect the tissue before placing the cap. If any tissue is mounded or folded, it is best not to place the cap over that area. Alternatively, the folded area of the tissue can be scraped off the slide using a sterile razor blade, leaving only flat portions of the tissue section. The tissue section must be dry and uncover-slipped during dissection. Next, enable the diode laser and visualize the tracking beam on the monitor as well as through the eyepieces. Once the laser is focused using the laser-tracking beam and then the focus of the tissue is adjusted using $\times 20$ objective, one is ready for dissection. Refocus the laser beam for each tissue section and slide at the $7.5\text{-}\mu\text{m}$ beam setting by turning the laser-focusing wheel until the tracking beam shows a bright spot with a well-defined edge. There should be no bright rings surrounding the central spot. There is no need to refocus the $15\text{ }\mu\text{m}$ or $30\text{ }\mu\text{m}$ beams. They are automatically calibrated once the $7.5\text{-}\mu\text{m}$ beam is focused. Adjust the laser power and pulse duration settings for the particular spot size following the manufacturer's recommendation and as needed to obtain good tissue "wetting" by molten plastic, as indicated by clear areas formed with each laser pulse. If the edges of the area of clearing are not well delineated, check to make sure the tissue section (where the cap is placed) is flat. If this fails to correct the problem, we recommend increasing the power and/or duration gradually, testing for wetting with higher energy/duration. Move the stage with the joystick, targeting selected cells by firing the button-actuated laser. When targeting is complete, lift the placement arm and inspect the area in which the laser was fired for removal of cells. Dissected areas should show near total removal of tissue. Quality of dissection should be checked occasionally by releasing the vacuum slide holder, moving the slide so that a clean area without tissue is in the microscopic field of view, lowering the cap to the slide, and scanning the surface of the cap. The microdissected tissue should be visible on the cap surface. If this is not the case, there are several explanations and potential remedies (*see* **Notes 52–61**). Avoid lifting and lowering the cap after firing the laser and capturing tissue. Already-captured tissue attached to the LCM cap will be placed on the histologic section away from its point of origin, resulting in a layering effect, which can limit contact of the cap with the tissue and compromise the effectiveness of LCM. However, before proceeding with a large-scale microdissection, we recommend that the user check for quality of dissection after several pulses. This should not interfere with subsequent dissection and the cap can be placed back in the same position. In addition, dense, dark, or thick samples may occlude the tracking beam. If this occurs, the intensity of

the tracking beam should be increased. Once LCM is achieved successfully with initial test pulses, the remainder of the desired cells can be efficiently microdissected.

3.8.1.3. COLLECTION OF THE MICRODISSECTED CELLS

After the microdissection is completed, the placement arm with the cap is moved to the “unload platform” and, using the cap insertion tool, the cap is removed. Cells that were not selected for capture may stick to the surface of the cap at random, and it is important to remove this unwanted tissue. This can be accomplished by using CapSure Pads®, purchased from the manufacturer, which have a sticky surface. A less costly alternative to the CapSure Pad is to use the sticky surface of Post-it® Notes, which can be used after the cap has been removed from the unload platform (gently touch the cap three separate times to clean areas of the Post-it Note, then view the cap microscopically to ensure that all loose material is removed). To obtain successful removal of unwanted cells, one may have to repeat this two or three times. When removal of unwanted material is complete, use the cap insertion tool to place the LCM cap onto a Perkin Elmer/Applied Biosystems GeneAmp 500-μL thin-walled PCR reaction tube (cat. no. N8010611) containing the appropriate amount of lysis buffer. The buffer used will depend upon the analyte, for example, RNA, DNA, or protein, and the method of analysis. Do not seat the LCM cap fully on the microcentrifuge tube, as this will cause undue stretching and possible reagent leakage. Leave a 2-mm gap between the cap top and the rim of the microcentrifuge tube to avoid this problem. For best results, use only Eppendorf tubes, as they are well-matched to the size of the lower portion of the CapSure™ caps. Invert the tube so that lysis buffer contacts the cap surface, and place on ice or refrigerate until the microdissection session is over, to preserve the analyte. If regular CapSure™ caps are used (not HS caps described later), an exposed blank LCM cap control is recommended for each experiment to ensure that nonspecific transfer is not occurring during microdissection. This is best performed by placing an LCM cap on the tissue section being dissected and aiming and firing the laser at regions where there are no cells or structures present, for example, lumens of large vessels, cystic structures, and so forth (alternatively one can place a portion of the LCM cap “off” the tissue and target this region). The cap should be processed through the buffer and analysis methodology applied. This serves as a negative control.

3.8.2. LCM Method for Epithelial Cell Enrichment

Maitra et al. have described an enhancement to LCM that can be used to enrich samples for neoplastic cells (45). This method, called “epithelial aggregate separation and isolation,” or EASI, is applicable to fresh tissues only.

1. The tissue is sectioned and gently scraped with the edge of a plain, uncharged, microscope glass slide.
2. The material adherent to this slide is then spread evenly onto the surface of a second uncharged slide.
3. Slides are immediately fixed in 95% methanol for 2 min and stained with H&E.
4. Epithelial aggregates on these slides can then be microdissected using an LCM. Alternatively, manual methods can also be used.
5. The advantages of this technique are that the discreteness of the epithelial clusters helps reduce background inflammatory and stromal elements and that large areas can be sampled (46).

3.8.3. LCM Dissection of Single or Small Numbers of Cells

Arcturus Engineering has developed a line of related products specially designed for high sensitivity capture and extraction of a single cell or a small number of cells. There are three key components of the system: (1) a preparation strip that flattens the tissue section and removes loose debris, (2) a high-sensitivity transfer cap (CapSure HS™ cap) that keeps the cap surface out of contact with the untargeted section surface, and (3) a low-volume reaction chamber that fits onto the high sensitivity transfer caps and accepts a low volume of lysis or digestion buffer while sealing out any nonselected material from the captured cells. The surface coated with polymer contacts only the tissue in the area that the laser is fired, thus reducing nonspecific transfer of tissue. Using the HS caps, it is preferable to capture cells as close to the center of the cap as possible. Unlike basic LCM using standard caps, the HS caps can be repositioned as often as needed to keep the targets toward the center of the cap, the cap surface does not contact the tissue except at the area that the laser is fired. It is important to stay within the capture ring because areas outside the ring will be excluded from the low-volume reaction tube. After the intended microdissection is completed, the HS cap is placed on the unload platform, picked with the cap insertion tool, and placed into the alignment tray. The specialized low-volume reaction chamber is then positioned over the cap. The chamber has a port for insertion of the lysis/digestion buffer. Ten microliters of the desired buffer is delivered into the fill port, which is covered securely with a thin-walled Gene-Amp PCR tube.

3.8.4. PALM Microbeam System

Follow the manufacturer's instructions for general use of the instrument.

3.8.5. Leica LMD System

As for the PALM system, follow the manufacturer's instructions for general use of the instrument.

3.9. Isolation of Analyte from Microdissected Material

3.9.1. Isolation of DNA

3.9.1.1. KIT-BASED METHOD (BASED ON DNEASY KIT BY QIAGEN)

(This protocol applies to all types of tissue material microdissected from glass slides. Ignore references to LCM caps if material was manually dissected or dissected using other devices).

1. Remove the LCM Cap using the cap-removal tool, add 45 μ L of proprietary Buffer ATL and 5 μ L of proteinase K mixture (45:5) per cap, and replace the cap, taking care not to fully seat the cap (causes stretching and later leakage).
2. Mix the solution by vortexing briefly, flick down the tube in an upside down position so that the cells on the cap make contact with the solution, vortex five times at 1-s intervals, and incubate at 55°C for 3 h in a Hybaid oven or other oven that provides gentle rocking motion. Vortex again three times.
3. Centrifuge the tubes at 5000 rpm (2700g) for 5 min at room temperature in a micro-centrifuge.
4. Heat tubes at 95°C at 600 rpm using a Thermomixer R (Eppendorf) or other heating device for 10 min to inactivate proteinase K (if sample is to be used directly for PCR).
5. Add 2 μ L of 0.5 mg/mL of RNase A, vortex briefly, and incubate at 37°C for 30 min.
6. Combine all samples from each case (if multiple caps per unique case) into a fresh microcentrifuge tube, add 150 μ L of proprietary buffer AL, immediately mix by vortexing, and incubate at 70°C for 10 min. (White precipitate may form after addition of AL buffer, but will dissolve while incubating at 70°C.)
7. Add 150 μ L of room temperature 100% ethanol and mix thoroughly by vortexing.
8. Transfer the sample into a DNeasy mini-column sitting in the provided 2-mL collection tube and centrifuge at 8000 rpm (6800 g) for 1 min at room temperature.
9. Transfer the column into a fresh collection tube, add 500 μ L of buffer AW1, and centrifuge for 1 min at the same speed.
10. Transfer the column into a fresh collection tube, add 500 μ L of buffer AW2, and centrifuge for 3 min at 17,900g (13,000 rpm with Eppendorf 5417C microcentrifuge, using standard fixed angle rotor) to dry the column.
11. To elute DNA from the column, transfer the column into a 1.5-mL microcentrifuge tube, add 200 μ L of buffer AE, and centrifuge for 1 min at 10,600g (10,000 rpm with Eppendorf 5417C microcentrifuge, using standard fixed angle rotor). To increase recovery of the DNA, use the buffer containing the DNA after the first elution to elute the column one more time.
12. Add 1 μ L of 20 mg/mL glycogen, 20 μ L of 3 M sodium acetate, pH 5.2, 440 μ L of 100% ethanol, mix by vortexing and incubate at -20°C for at least 1 h (we are unable to find a reference supporting an optimal time for precipitation).
13. Centrifuge the sample at 13,000 rpm at +4°C for 30 min, aspirate the supernatant, and resuspend the pellet in 1 mL of 70% ethanol. Centrifuge the tube for 10 min, aspirate ethanol, and air-dry the pellet for 5–10 min.

14. Resuspend each DNA pellet in 5 μL of loTE buffer, pH 7.4 (see recipe below), combine all the sample in one tube and use 0.5 μL of the sample to quantitate using pico-green (or other) assay.

3.9.1.2. SDS/PHENOL-CHLOROFORM EXTRACTION

1. Transfer samples to a first SST tube. (This can be performed in 1.5-mL microcentrifuge tubes if volume requirements are smaller.)
2. Add 6 mL of phenol-chloroform-isoamyl alcohol (25:24:1) to each tube (*see Note 62*).
3. Vortex five times, setting 8.
4. Centrifuge at 2000g (STH-750 rotor), 20 min, room temperature.
5. Transfer the top (aqueous) layer to a second SST tube.
6. Add 6 mL of phenol-chloroform-isoamyl alcohol (25:24:1) to each tube.
7. Vortex five times (VWR vortex Genie, setting 8).
8. Centrifuge at 2000g, 20 min, room temperature.
9. Transfer the aqueous layer to a third SST tube.
10. Add 6 mL of phenol-chloroform-isoamyl alcohol (25:24:1) to each tube.
11. Vortex five times, setting 8.
12. Centrifuge at 2000g, 20 min, room temperature.
13. Transfer the aqueous layer to a 50-mL first sterile blue top conical tube.
14. Add 5.75 mL of chloroform-0.25 mL of isoamyl alcohol to each tube.
15. Vortex five times, setting 8.
16. Centrifuge at 2000g (3100 rpm in Sorvall Super T21 Refrigerated Centrifuge ST-H750 rotor, swing buckets), 20 min, at room temperature.
17. Transfer the aqueous phase to a second 50-mL conical tube.
18. Add 2.5 mL of ammonium acetate, mix gently.
19. Add 15 mL of “frozen” 100% ethanol, mix gently.
20. Store in -20°C , overnight.
21. Centrifuge at 3800g (4300 rpm in Sorvall Super T21 Refrigerated Centrifuge ST-H750 rotor, swing buckets), 30 min, 4°C .
22. Discard the supernatant gently into a waste flask, preserving pellet.
23. Wash pellet with 10 mL of 70% EtOH (room temperature), set for 10 min.
24. Centrifuge at 3800g, 10 min, 4°C .
25. Discard the supernatant into the waste flask.
26. Wash with 10 mL of 70% EtOH (room temperature), set for 10 min.
27. Centrifuge at 4300 rpm (STH-750 rotor), 10 min, 4°C .
28. Gently discard the supernatant into the waste flask.
29. Inverted at a 30° angle, air dry for $25 \pm \text{min}$ (do not overdry or DNA will not go into solution).
30. Resuspend in 200–1000 μL or more of LoTE (volume dependent on predicted yield and desired storage concentration), and store at 4°C , overnight.
31. Gently mix DNA and transfer DNA to a microcentrifuge tube or other storage device.

3.9.2. Isolation of RNA (see **Notes 63** and **64**)

We have obtained high-quality total RNA from microdissected samples using a phenol–chloroform-based approach, and also using the PicoPure RNA isolation kit (Arcturus), a column-based approach that is optimized for use with microdissected cells. Several other kit-based methods for isolation of total RNA from small tissue samples are currently on market from companies such as Ambion, Qiagen, and others (47), but we have not had the opportunity to test them. The phenol–chloroform and Arcturus kit-based methods are covered here.

Prior to performing total RNA extraction of any kind, it is wise to clean all pipettors with a RNase removal product such as RNase AWAY™ (Molecular BioProducts) or RNase ZAP™ (Ambion, Austin, TX).

3.9.2.1. KIT-BASED METHOD (PICO PURE RNA ISOLATION KIT BY ARCTURUS)

1. Dispense extraction buffer (XB) and incubate as follows:
 - a. Pipet 50 μ L of well-mixed XB into a Perkin Elmer/Applied Biosystems GeneAmp 500- μ L thin-walled PCR reaction tube (cat. no. N8010611) 0.5-mL microcentrifuge tube. This is the only type of tube that Arcturus currently recommends using with the original non-LCM caps currently, as other tubes vary in diameter and may be more prone to leakage.
 - b. Place CapSure LCM cap onto the microcentrifuge tube using an LCM cap insertion tool.
 - c. Invert the CapSure Cap-microcentrifuge tube assembly. Tap the microcentrifuge tube to ensure all XB is covering the CapSure LCM cap.
 - d. Incubate assembly for 30 min at 42°C.
2. Centrifuge assembly at 800g (approx 3500 rpm) for 2 min to collect cell extract.
3. Remove the LCM cap and save the tube with the extract in it.
4. Proceed with the RNA isolation or freeze the extract at –80°C.
5. Precondition the RNA purification column:
 - a. Pipet 250 μ L conditioning buffer (CB) onto the purification column filter membrane.
 - b. Incubate the column with conditioning buffer at RT for 5 min.
 - c. Centrifuge the purification column in the provided collection tube at 16,000g (approx 14,000 rpm) for 1 min.
6. Pipet 50 μ L of 70% ethanol (EtOH) into the cell extract from **step 4**. Mix well by pipetting. Do not centrifuge.
7. Pipet the cell extract and EtOH mixture into the preconditioned purification column. The combined volume will be approx 100 μ L.
8. Centrifuge for 2 min at 100g (approx 1200 rpm), immediately followed by 16,000g (14,000 rpm) for 30 s.
9. Pipet 100 μ L of wash buffer 1 (W1) into the purification column and centrifuge for 1 min at 16,000g (approx 14,000 rpm). A DNase step may be performed at this point:
 - a. DNase treatment (Qiagen RNase-free DNase Set, cat. no. 79254).

- b. Pipet 5 μL of DNase I stock solution into 35 μL of buffer RDD. Mix by inverting.
- c. Pipet the 40 μL of DNase incubation mix directly onto the purification column membrane.
- d. Incubate at RT for 15 min.
- e. Pipet 40 μL of PicoPure RNA Kit wash buffer 1 (W1) into the purification column. Centrifuge at 8000g (approx 10,000 rpm) for 15 s.
10. Pipet 100 μL wash buffer 2 (W2) into the purification column and centrifuge for 1 min at 16,000g (approx 14,000 rpm).
11. Pipet another 100 μL of wash buffer 2 into the purification column and spin for 2 min at 16,000g (14,000 rpm). Check the column for any residual wash buffer. If present, centrifuge for an additional minute.
12. Transfer the purification column to a new microcentrifuge tube provided in the kit.
13. Pipet 11 μL (maximum is 30 μL) elution buffer (EB) directly onto the membrane of the purification column.
14. Incubate the purification column for 1 min at RT and then centrifuge at 1000g (approx 3800 rpm) for 1 min and then maximum speed for 1 min to elute RNA. Use RNA immediately or store at -80°C until use.

3.9.2.2. PHENOL–CHLOROFORM-BASED RNA EXTRACTION METHOD

This method is modified from Chomczynski and Sacchi (48):

1. Ensure an RNase-free environment, including reducing likelihood that pipettors contain RNases as described in the preceding.
2. Dissolve tissue obtained by microdissection by placing in 200 μL of *GITC denaturing solution*. Invert several times over the course of 2 min to digest the tissue off the cap. If tissue does not dissolve completely, use an appropriate disaggregating device to homogenize.
3. For specimens that originated from paraffin-embedded tissue, it is helpful to include an incubation step (with the tube inverted) of 20 min at 60°C to further liberate the RNA from the tissue.
4. Remove the solution from the reagent tube and replace it in a sturdy RNase-free 1.5-mL microcentrifuge tube (cap must seal tightly, and must be able to withstand centrifugation at 10,000g; *test this if necessary*).
5. Add 20 μL (0.1X volume) of 2 M sodium acetate, pH 4.0.
6. Add 220 μL (1X volume) of water-saturated phenol.
7. Add 60 μL (0.3X volume) of chloroform–isoamyl alcohol.
8. Shake the tube vigorously for 15 s.
9. Place on wet ice for 15 min.
10. Centrifuge at 10,000g for 30 min at 4°C to separate the aqueous and organic phases.
11. Transfer the upper aqueous layer to a fresh tube (*see Note 65*).
12. Add to aqueous layer, 1–2 μL of glycogen (10 mg/mL) and 200–300 μL of cold isopropanol (i.e., equal volume). Glycogen facilitates visualization of the pellet, which can be problematic when using small amounts of RNA.

13. Place samples at -80°C (some use -20°C) for at least 30 min. It may be left overnight.
14. Before centrifuging, the tubes may need to be thawed slightly if they have solidified during the isopropanol precipitation.
15. Centrifuge for 30 min at 4°C with cap hinges pointing outward so that the location of the pellet can be better predicted.
16. Remove the supernatant and wash with 300 μL of cold 70% ethanol. Add the alcohol and centrifuge for 5 min at 4°C .
17. Remove the supernatant.
18. Let the pellet air dry on ice to remove any residual ethanol. Overdrying prevents the pellet from resuspending easily (drying is not necessary however if the RNA is to be resuspended in formamide).
19. The pellet may be stored at -80°C until use or proceed to DNase treatment (*see below*).
20. Dissolve the RNA pellet in 10–20 μL DEPC-treated water and store at -80°C , or dissolve in a similar amount of deionized formamide by passing the solution a few times through a pipett tip and store at -20°C or -80°C .

3.9.2.3. DNase TREATMENT OF TOTAL RNA

(ALTERNATE TO METHOD IN **SUBHEADING 3.9.2.1., STEPS 9A–9E**)

1. DNase treatment is highly recommended for microdissected cells. Genomic DNA contamination is often problematic with these samples, possibly due to the small DNA fragments that are created during tissue processing and are difficult to purify from RNA.
2. To an RNA pellet, add 15 μL of DEPC-treated water and 1 μL (20 U/ μL) RNase inhibitor (Perkin Elmer).
3. Gently mix by flicking until the pellet is dissolved.
4. Pulse spin on microcentrifuge.
5. Add 2 μL of 10X DNase buffer (GenHunter) and 2 μL (10 U/ μL) DNase I (GenHunter; 20 U total).
6. Incubate at 37°C for 2 h.
7. Reextract RNA by adding:
 - a. 2 μL 2 M sodium acetate, pH 4.0.
 - b. 22 μL of water-saturated phenol.
 - c. 6 μL of chloroform-isoamyl alcohol.
8. Shake vigorously for 15 s.
9. Place on wet ice for 5 min.
10. Centrifuge at 10,000g for 10 min at 4°C .
11. Transfer upper layer to a fresh tube.
12. Continue with RNA extraction from **step 12** in **Subheading 3.9.2.2.**, adjusting the volume of isopropanol accordingly.

4. Notes

1. Commercial preparations of 10% neutral buffered formalin contain 1–10% methanol (**13**). This is added to inhibit polymerization of formaldehyde, which can grad-

ually precipitate out of solution. Commercially prepared NBF is not used for electron microscopy because the added methanol can cause coagulation of proteins. Similarly, molecular laboratories wishing to avoid protein coagulation should consider making formaldehyde solutions from scratch for this reason.

2. Formaldehyde is a carcinogen in rodents and can induce allergies in humans. Use appropriate means to avoid inhalation and contact exposure under all circumstances.
3. A 37% formaldehyde solution can be prepared by dissolving 37 g of paraformaldehyde powder (e.g., Sigma) in 100 mL of water in a fume hood. Heat to 70°C (no higher—to avoid decomposition) to allow paraformaldehyde to dissolve and dissociate into a mixture of polymeric and nonpolymeric formaldehyde, and cool to room temperature. Note that many laboratories call any fixative made directly from paraformaldehyde powder “paraformaldehyde,” which is something of a misnomer because all formaldehyde solutions are a mixture of formaldehyde and paraformaldehyde.
4. The term “formalin” should be abandoned because of the confusion it causes, but nonetheless is in common usage. “10% formalin” by tradition is what is obtained when 37% formaldehyde is diluted 1:10. The correct term is 3.7% formaldehyde.
5. The resulting pH of this 10% neutral buffered formalin solution is approx 6.8, and is hypotonic at approx 165 mosM. Isotonic 3.7% formaldehyde can be prepared by increasing sodium phosphate monobasic to 18.6 g, eliminating sodium phosphate dibasic, and adding 4.2 g of NaOH. This is known as Modified Millonig Formalin, and isotonic (310 mosM), with a pH of 7.2–7.4. It can be used both for histologic preparation of tissues for light and electron microscopy (12). Note that formaldehyde itself is reported not to be osmotically active (49).
6. Molecular studies performed in our laboratories have shown that both 70% ethanol and a 4.2:1.8:2.0 (v:v:v) of ethanol–methanol–water provide excellent histologic detail, and markedly improved DNA (7), RNA (7), and protein quality (4) compared to standard 10% NBF fixation.
7. 70% Ethanol is easy to prepare, but may be open to abuse (human consumption) in some laboratory environments.
8. The final concentration of ethanol in this mixture is 52.5%, and the final concentration of methanol is 25%. The mixture needs to be marked as toxic, as it can cause blindness if ingested.
9. Methanol penetrates tissues measurably faster than ethanol in our studies (G. S. B., unpublished data).
10. In a blinded survey of practicing pathologists, kidney and prostate histology were better with this ethanol–methanol mixture than with 70% ethanol, although both were rated acceptable (7).
11. Complete proteinase K digestion is critical to downstream DNA isolation efficiency. If proteins are inaccessible to proteinase K because of residual paraffin, or other hydrophobic compounds, DNA recovery will be affected.
12. Even when proteins are accessible to the enzyme, incomplete digestion will also markedly reduce yield. Well-digested samples are clearer, without clumps, and flow freely. Add more proteinase K and incubate longer if digestion appears incomplete.

Proteinase K functions at least between 37°C and 55°C, and may function at room temperature, although we have not tested this. We use 48°C, and this works.

13. Direct immersion of tissue in liquid nitrogen often leads to poor histological preservation because freezing is not rapid enough to prevent formation of ice crystals large enough to damage cell morphology, and should be avoided. A jacket of insulating nitrogen gas forms around the tissue after immersion, slowing heat transfer. Two methods that provide more rapid freezing and better histology are discussed.
14. Tissues thicker than roughly 2 mm tend to crack, probably because of stresses caused by differential expansion of the tissue as the wave of freezing passes through the tissue from outside to inside.
15. Multiple blocks can be frozen simultaneously if the basket is large enough
16. If freezing in OCT is desired, plastic cryomolds can be purchased for this purpose. Tissues frozen at -80°C or below for long periods with no covering oil or other material become severely dessicated ("freezer burn"), which, however, may also occur in samples stored in OCT over longer periods. Instrumedics (Hackensack, NJ) sells oil for covering tissues to prevent desiccation, but we do not have independent confirmation that this technique works.
17. This is our preferred method for rapid freezing, because it does not require use of flammable liquid, and in our hands (G. S. B.) provides histological quality as good as standard H&E-stained formalin fixed paraffin-embedded sections.
18. Only one block can be frozen at a time (taking 1 min or so per block) using this device and if many blocks need to be frozen simultaneously, this method can be prohibitively slow.
19. The device can be used without an embedding compound, but may cause the tissue to stick to the piston or to the base. We always use an embedding compound, and cut clear plastic from a report cover to approx 3 cm × 3 cm, cover this with 4 mm of embedding compound, freeze enough to make firm (but not crack) and use this as a base on which to place tissue to be frozen. A layer of embedding media is put over the entire top and sides of the tissue, and the cold metal piston is then lowered onto the tissue.
20. The two greatest advantages of disaggregation of tissues are that living cells can be obtained and that whole cells are recoverable. The disadvantages are that tissue morphology is lost, and a reliable molecular method (usually antibody-specific staining) is required to identify the cells of interest after disaggregation.
21. The same basic caveats apply to cytologic specimens as to histologic sections: alcohol fixation is preferred, especially for RNA analysis.
 - a. Smeared cells should not be allowed to dry on the slide prior to fixation, particularly for those using LCM (difficult to remove cells from slide surface). Fixed and stained cells should be adequately dehydrated prior to attempting LCM.
 - b. We prefer to prepare cytologic smears with a hemocytometer cover (rather than using a separate glass slide) because the width is slightly less than that of the standard glass microscopic slide and the resulting smear (and cells) are not spread to the edge (or off) of the slide, where they are difficult or impossible to stain and microdissect.

22. As for direct smears of liquids containing suspended cells, smeared cells should not be allowed to dry on the slide prior to fixation, particularly for those using LCM (difficult to remove cells from slide surface). Fixed and stained cells should be adequately dehydrated prior to attempting LCM.
23. This technique works best when target cells are less adherent to tissue than non-target cells. Most epithelia are less adherent than underlying stroma for example.
24. Density and size-based separation techniques traditionally has been used to separate blood mononuclear cells, cell organelles, and microorganisms, but has not been used routinely for disaggregated solid tissue cell separation, because of the time the cells spend unfixed and because of the loss of tissue morphology. Theoretically, these problems are surmountable through isosmotic fixation of cells, and if the cells are phenotypically distinct based on a specific antibody or other marker, this method may become more useful as specific phenotypic immunomarkers and other biomarkers are identified. An advantage of this method when compared to tissue sectioning is that whole cells are obtained.
25. A large amount of pertinent information about magnetic bead-based separations is contained in the Dynal website (www.dynalbiotech.com). Other manufacturers also offer bead-based separation systems.
26. Cytospin[®] preparations can be used for any cytologic sample but are preferred for samples of low cellularity. Another alternative to handling samples of low cellularity is to centrifuge the sample, pour off the supernatant, and make a direct smear from the sediment concentrated in a low volume of liquid. Particularly bloody specimens may benefit from Ficoll separation. If such a separation is used, to avoid RNA, DNA, or protein degradation, the cytologic samples should be processed and fixed in 95% ethanol before processing.
27. This method was originally developed for electron microscopy, and can provide improved histology over standard frozen section techniques, and theoretically will provide biomolecular integrity intermediate between frozen and formalin-fixed, paraffin-embedded tissue. We are not aware of its use in molecular studies to date, but it warrants testing for molecular profiling studies requiring excellent histology, moderate biomolecule quality, and avoidance of the relatively high temperature exposures of standard tissue processing. It is also notable that since proteins should not be denatured using this technique, and because heating (antigen retrieval) can reverse formaldehyde crosslinks, native proteins may be obtainable using this technique.
28. It is notable that an effort is currently underway by Sakura Finetek (Torrance, CA) and the University of Miami School of Medicine to replace current standard surgical pathology tissue processing methods with the goal of reducing time and to increase the molecular preservation and analyzability of samples (50).
29. Frozen sections allowed to come to room temperature and dry prior to staining will usually adhere too strongly to the slide, preventing effective LCM. Other types of microdissection are not affected by drying.
30. If frozen tissue sections are to be used for RNA analysis, it is essential that they be stored at -80°C and used within 2–3 mo of sectioning. This conclusion is based

- an anecdotal experience in which tissue microdissected from frozen sections on slides stored for 2 yr did not yield high quality RNA (further study may be needed).
31. If slides are to be stored in a slide file, allowed to cool first. Storage in slide boxes with space between slides is probably optimal to reduce risk of damage to sections and contamination, but this has not been studied to our knowledge.
 32. The need for DEPC-treated water in the section-floating bath to eliminate RNase exposure and/or use of protease inhibitors in the water bath to eliminate protease exposure has not been tested to our knowledge. Immunostaining of paraffin sections floated in ordinary tap water has been the rule for several decades, so it is likely that paraffin inhibits significant protein degradation, but depending on your applications, testing under controlled conditions may be warranted for potential effects of proteases, especially on low-abundance proteins.
 33. Use the least amount of Mayer's hemalum (MH) and eosin necessary for visualization of the cells of interest. Recovery of DNA, RNA, and protein is improved as MH and eosin content decreases. Decreased stain content can be achieved by decreasing time of exposure or by decreasing concentration of stain used. Decreased concentration (we use 10% of normal) provides greater control if staining is done manually.
 34. Minimized MH intensity in sections also allows better visualization during microdissection, because MH-stained areas appear much darker than normal when no coverslip is in place. MH-stained areas in "normally" MH&E stained tissue sections appear black when no cover slip is present because of light scattering at the tissue-air interface (scattering occurs because of refractive index mismatch between air and tissue).
 35. Poor LCM (Arcturus PixCell device) transfers will occur if sections are not fully dehydrated and xylene treated. Be sure that the 100% ethanol used are fresh to ensure dehydration. Xylenes should be changed when cloudy.
 36. The final xylene step is necessary for successful Arcturus LCM, but may not be necessary for other types of microdissection. Xylene treatment appears to remove the alcohols more effectively, allowing the LCM EVA plastic to adhere better to the tissue, although this is not proven. When using membrane-coated slides (with PALM, Leica devices) with xylene, test blank membrane-coated slides in xylenes prior to staining sections. Membrane formulations are changing, and some membranes may dissolve in xylenes.
 37. The mRNA recovered from tissue with a short blood perfusion-free interval and that is rapidly frozen and immunostained is generally of high quality. Single-step PCR allows amplification of fragments of more than 600 bp from both housekeeping genes, for example, β -actin, as well as cell-specific messages, for example, CD4 or CD19, using cDNA derived from less than 500 immunostained, microdissected cells (NCI Laboratory results).
 38. For primary antibodies other than those included in a commercial kit such as in DAKO Quick Staining kit, the dilutions should be determined individually. Add placental RNase inhibitor to the primary antibody and the DAB solution in a concentration of 200–400 U/mL. All solutions are prepared with DEPC-treated water.

39. If RNA is to be isolated from dissected material, it is essential that microdissection proceed immediately after slide preparation since significant RNA degradation may occur in fully dehydrated tissue sections after just one hour at room temperature. In addition, captured cells should be extracted with GITC (guanidinium isothiocyanate) buffer as soon as possible.
40. PBS recipe:
 - a. Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂PO₄, and 0.24 g of KH₂PO₄ in 800 mL of distilled H₂O.
 - b. Adjust pH to 7.4 with HCl.
 - c. Adjust volume to 1 L with additional distilled H₂O.
 - d. Filter sterilize, or sterilize by autoclaving.
41. Stains nuclei dark blue, stains cytoplasm where there are concentrations of RNA. As stated in the introduction, methylene blue staining was associated with poor RNA recovery in one non-peer-reviewed but credible study (16).
42. Stains DNA and RNA greenish blue. Provided best RNA recovery in the same study mentioned earlier (16).
43. Stains nuclei dark red, cytoplasm lighter red. Performed as well as H&E for RNA recovery in the same study (16).
44. Be sure to trim away OCT or other mounting medium as much as possible as it can interfere with downstream protocols.
45. Dissected sections can be retained in 50-mL conical tubes at -80°C for long periods prior to further processing.
46. If sections need to be placed directly in digestion buffer for DNA, RNA, or protein isolation, carefully place frozen sections in the bottom of the tube (50-mL conical tube for large volume of sections, or microcentrifuge tube for small volume of sections), and use gentle mechanical rocking to get all the sections to go into solution.
47. If no micromanipulator arm is available, the dissector should prop his or her elbow on a solid surface adjacent to and at the same height as the stage of the microscope to stabilize the dissecting hand. It is helpful to rest the ulnar aspect of the dissecting hand on the stage of the microscope and move the needle into the microscopic field, a few millimeters above the tissue. In this way, the dissecting arm and hand can be rested on solid support surfaces.
48. Pressing down on the shaft of the syringe to inject an air bubble into the extraction solution helps to detach the tissue from the needle and prevents any fragments from remaining lodged in the barrel of the needle.
49. Placement of frozen tissue sections directly on agarose coated slides can be helpful in maintaining enzyme stability if the investigator wishes to recover proteins in native form from a frozen section. In addition, agarose gels can be prepared or soaked in custom buffers that will bathe the frozen section prior to and during the microdissection, for example, pH, salt concentration, proteinase inhibitors, and so forth, can be varied specifically for a given enzyme. Some members of the NCI group also prefer to use the agarose-coated slide microdissection approach for recovery of mRNA.

50. Slides for microdissection are prepared by placing 200 μ L of warm agarose on standard uncoated glass slides, covering with a glass slip, and allowing the gel to polymerize. The glass slip is removed from the slide and the frozen tissue section is immediately placed onto the agarose gel. For best results, the freshly cut section should be transferred directly from the cryostat to the agarose-coated slide.
51. The dissector may find it easier to “tease” the tissue apart since the tissue remains bathed in the fluid from the gel and can be gently pulled apart. The tissue will also separate along tissue planes, for example, stroma and epithelium will easily separate from each other. The dissected tissue can be gently picked up from the slide or, alternatively, the dissector can use a needle to physically cut the agarose and procure both the agarose and the tissue fragment together.
52. Ensure that the slide is completely free of xylene. Use a Cleantex Microduster (MG Chemicals, others) or other drying mechanism if necessary to dry the slide thoroughly.
53. Ensure that tissue sections are flat. Wrinkles can be shaved off with sterile razor blades. The section should be dipped in xylene after shaving the wrinkles to ensure that no contaminating debris remains on the section.
54. Ensure that silver metal weight on cap support arm is resting freely on slide. Add additional weight temporarily.
55. Refocus the laser beam. Increase laser power and/or pulse duration.
56. Change the cap. Not all caps perform equally. Cap shelf-life may be important (but no good data on this is readily available). We recommend buying relatively small numbers of caps so that one’s stock remains relatively new.
57. Repeat dehydration of the specimen with fresh xylenes. Submerge for 1 min or more and allow drying in hood for 1–5 min. If LCM is still not successful, pass the slides through 95% alcohol for 30 s twice, absolute alcohol for 30 s twice, and then xylenes for 1–5 min.
58. Cut a new tissue section onto a new glass slide, ensuring that the frozen sections or cytologic specimens have not been allowed to dry on the slide prior to fixation. For formalin-fixed sections, either do not bake or decrease the baking time.
59. Try a different brand or type of glass slide.
60. Check lab humidity. Under highly humid conditions, water may be transferred to the tissue rapidly, reducing EVA adhesion to tissue. Attempt to lower humidity if this could be a problem.
61. If still not successful, talk with other researchers to continue to troubleshoot the problem. In addition, Arcturus Engineering technical support (easily available on the WWW) has been very helpful in many situations.
62. Phenol–chloroform–isoamyl alcohol (25:24:1) can be prepared in the laboratory, but take great care to handle phenol only in a fume hood and with appropriate protective gear, as it can cause severe burns. Phenol melts at 41°C and boils at 61°C. Phenol must be appropriately pH-buffered prior to mixing.
63. It is estimated that a typical mammalian cell contains 2 pg of total RNA per cell, therefore, to achieve 5 μ g of total RNA, the lower limit for some expression arrays,

will require the microdissection of 2.5 million cells, a daunting task. Therefore, some authors have advocated amplification of RNA or resultant cDNA prior to hybridization with these larger arrays, even though this may introduce some degree of amplification bias.

64. The duration of the actual microdissection session on each frozen section should be limited to 15–30 min for optimal RNA preservation. Samples for protein analysis are also best processed as for RNA analysis, but reagents should include protease inhibitors.
65. If any of the lower organic phase was accidentally transferred during RNA isolation and may be contaminating the aqueous phase, this will interfere with the subsequent isopropanol precipitation. To remove any residual organics from the aqueous layer, add one volume of 100% chloroform, mix well, and centrifuge for 10 min at 4°C to again separate the aqueous and organic phases. Transfer the upper layer to a new tube.

Acknowledgments

Special thanks to Drs. Eun-Chung Park, Douglas P. Clark, and Anirban Maitra for critical reading and suggestions for improvement of this manuscript, and to Steven H. Chen for technical assistance in preparation of the manuscript.

Portions of the text are adapted from Best and Emmert-Buck (3) with permission of the publisher Future Drugs Ltd. (London, England). Figures 2–5 are adapted from Eltoum, Siegal and Frost (1) with permission of Lippincott Williams & Wilkins (Philadelphia).

References

1. Eltoum, I. A., Siegal, G. P., and Frost, A. R. (2002) Microdissection of histologic sections: Past, present, and future. *Adv. Anat. Pathol.* **9**, 316–322.
2. Srinivasan, M., Sedmak, D., and Jewell, S. (2002) Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am. J. Pathol.* **161**, 1961–1971.
3. Best, C. J. and Emmert-Buck, M. R. (2001) Molecular profiling of tissue samples using laser capture microdissection. *Expert. Rev. Mol. Diagn.* **1**, 53–60.
4. Ahram, M., Flaig, M. J., Gillespie, J. W., et al. (2003) Evaluation of ethanol-fixed, paraffin-embedded tissues for proteomic applications. *Proteomics* **3**, 413–421.
5. Englert, C. R., Baibakov, G. V., and Emmert-Buck, M. R. (2000) Layered expression scanning: Rapid molecular profiling of tumor samples. *Cancer Res.* **60**, 1526–1530.
6. Fend, F., Emmert-Buck, M. R., Chuaqui, R., et al. (1999) Immuno-LCM: Laser capture microdissection of immunostained frozen sections for mRNA analysis. *Am. J. Pathol.* **154**, 61–66.
7. Gillespie, J. W., Best, C. J., Bichsel, V. E., et al. (2002) Evaluation of non-formalin tissue fixation for molecular profiling studies. *Am. J. Pathol.* **160**, 449–457.
8. Gillespie, J. W., Ahram, M., Best, C. J., et al. (2001) The role of tissue microdissection in cancer research. *Cancer J.* **7**, 32–39.

9. Ornstein, D. K., Gillespie, J. W., Paweletz, C. P., et al. (2000) Proteomic analysis of laser capture microdissected human prostate cancer and in vitro prostate cell lines. *Electrophoresis* **21**, 2235–2242.
10. Simone, N. L., Remaley, A. T., Charboneau, L., et al. (2000) Sensitive immunoassay of tissue cell proteins procured by laser capture microdissection. *Am. J. Pathol.* **156**, 445–452.
11. Bancroft, J. D. and Gamble, M. (2002) *Theory and Practice of Histological Techniques*, 5th ed. Edinburgh: Churchill Livingstone.
12. Carson, F. L. (2003) *Histotechnology: A Self Instructional Text*, 2nd ed. Chicago: ASCP Press.
13. Kiernan, J. A. (2001) *Histological and Histochemical Methods*, 3rd ed. Oxford: Oxford University Press.
14. Kiernan, J. A. and Mason, I. (eds.) (2002) *Microscopy and Histology for Molecular Biologists: A User's Guide*. London: Portland Press.
15. Histonet Listserver Information <http://www.histonet.org/>.
16. Gassmann, M. (2003) Quality Assurance of RNA derived from laser microdissected tissue samples obtained by the PALM(R) MicroBeam System using the RNA 6000 Pico LabChip(R) kit. 1-8. 2003. Agilent Technologies.
17. Horobin, R. W. and Kiernan, J. A. (eds.) (2002) *Conn's Biological Stains: A Handbook of Dyes, Stains and Fluorochromes for Use in Biology and Medicine*, 10th ed. Published for the Biological Stain Commission by BIOS Scientific Publishers, Distributed in US by Springer-Verlag (U.S.), Oxford, UK.
18. Zhuang, Z., Bertheau, P., Emmert-Buck, M. R., et al. (1995) A microdissection technique for archival DNA analysis of specific cell populations in lesions <1 mm in size. *Am. J. Pathol.* **146**, 620–625.
19. Lee, J. Y., Dong, S. M., Kim, S. Y., Yoo, N. J., Lee, S. H., and Park, W. S. (1998) A simple, precise and economical microdissection technique for analysis of genomic DNA from archival tissue sections. *Virchows Arch.* **433**, 305–309.
20. Gupta, S. K., Douglas-Jones, A. G., and Morgan, J. M. (1997) Microdissection of stained archival tissue. *Mol. Pathol.* **50**, 218–220.
21. Harsch, M., Bendrat, K., Hofmeier, G., Branschoid, D., and Niendorf, A. (2001) A new method for histological microdissection utilizing an ultrasonically oscillating needle: Demonstrated by differential mRNA expression in human lung carcinoma tissue. *Am. J. Pathol.* **158**, 1985–1990.
22. Beltinger, C. P. and Debatin, K. M. (1998) A simple combined microdissection and aspiration device for the rapid procurement of single cells from clinical peripheral blood smears. *Mol. Pathol.* **51**, 233–236.
23. Zhang, Z., Nakamura, M., Taniguchi, E., Shan, L., Yokoi, T., and Kakudo, K. (1997) A simple approach to single-cell microdissection and molecular analysis. *Anal. Quant. Cytol. Histol.* **19**, 514–518.
24. Emmert-Buck, M. R., Bonner, R. F., Smith, P. D., et al. (1996) Laser capture microdissection [see Comments]. *Science* **274**, 998–1001.
25. Curran, S., McKay, J. A., McLeod, H. L., and Murray, G. I. (2000) Laser capture microscopy. *Mol. Pathol.* **53**, 64–68.

26. Goldsworthy, S. M., Stockton, P. S., Trempus, C. S., Foley, J. F., and Maronpot, R. R. (1999) Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. *Mol. Carcinog.* **25**, 86–91.
27. Lawrie, L. C., Curran, S., McLeod, H. L., Fothergill, J. E., and Murray, G. I. (2001) Application of laser capture microdissection and proteomics in colon cancer. *Mol. Pathol.* **54**, 253–258.
28. Craven, R. A. and Banks, R. E. (2002) Use of laser capture microdissection to selectively obtain distinct populations of cells for proteomic analysis. *Methods Enzymol.* **356**, 33–49.
29. Nakazono, M., Qiu, F., Borsuk, L. A., and Schnable, P. S. (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.
30. Luzzi, V., Mahadevappa, M., Raja, R., Warrington, J. A., and Watson, M. A. (2003) Accurate and reproducible gene expression profiles from laser capture microdissection, transcript amplification, and high density oligonucleotide microarray analysis. *J. Mol. Diagn.* **5**, 9–14.
31. Burgemeister, R., Gangnus, R., Haar, B., Schutze, K., and Sauer, U. (2003) High quality RNA retrieved from samples obtained by using LMPC (laser microdissection and pressure catapulting) technology. *Pathol. Res. Pract.* **199**, 431–436.
32. Fink, L., Kohlhoff, S., Stein, M. M., Hanze, J., et al. (2002) cDNA array hybridization after laser-assisted microdissection from nonneoplastic tissue. *Am. J. Pathol.* **160**, 81–90.
33. Cohen, C. D., Grone, H. J., Grone, E. F., Nelson, P. J., Schlondorff, D., and Kretzler, M. (2002) Laser microdissection and gene expression analysis on formaldehyde-fixed archival tissue. *Kidney Int.* **61**, 125–132.
34. Kleeberger, W., Rothamel, T., Glockner, S., Lehmann, U., and Kreipe, H. (2000) Laser-assisted microdissection and short tandem repeat PCR for the investigation of graft chimerism after solid organ transplantation. *Pathobiology* **68**, 196–201.
35. Inoue, K., Sakurada, Y., Murakami, M., Shiota, M., and Shiota, K. (2003) Detection of gene expression of vascular endothelial growth factor and flk-1 in the renal glomeruli of the normal rat kidney using the laser microdissection system. *Virchows Arch.* **442**, 159–162.
36. Mori, M., Mimori, K., Yoshikawa, Y., et al. (2002) Analysis of the gene-expression profile regarding the progression of human gastric carcinoma. *Surgery* **131**, S39–S47.
37. Brockhoff, G., Fleischmann, S., Meier, A., Wachs, F. P., Hofstaedter, F., and Knuechel, R. (1999) Use of a mechanical dissociation device to improve standardization of flow cytometric cytokeratin DNA measurements of colon carcinomas. *Cytometry* **38**, 184–191.
38. Pertoft, H. (2000) Fractionation of cells and subcellular particles with Percoll. *J. Biochem. Biophys. Methods* **44**, 1–30.
39. Tai, Y. T., Teoh, G., Shima, Y., et al. (2000) Isolation and characterization of human multiple myeloma cell enriched populations. *J. Immunol. Methods* **235**, 11–19.

40. Moskaluk, C. A. and Kern, S. E. (1997) Microdissection and polymerase chain reaction amplification of genomic DNA from histological tissue sections. *Am. J. Pathol.* **150**, 1547–1552.
41. Goelz, S. E., Hamilton, S. R., and Vogelstein, B. (1985) Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochem. Biophys. Res. Commun.* **130**, 118–126.
42. Emmert-Buck, M. R., Gillespie, J. W., Paweletz, C. P., et al. (2000) An approach to proteomic analysis of human tumors. *Mol. Carcinog.* **27**, 158–165.
43. Ikeda, K., Monden, T., Kanoh, T., et al. (1998) Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. *J. Histochem. Cytochem.* **46**, 397–403.
44. Novelli, M., Savoia, P., Cambieri, I., et al. (2000) Collagenase digestion and mechanical disaggregation as a method to extract and immunophenotype tumour lymphocytes in cutaneous T-cell lymphomas. *Clin. Exp. Dermatol.* **25**, 423–431.
45. Maitra, A., Wistuba, I. I., Virmani, A. K., et al. (1999) Enrichment of epithelial cells for molecular studies. *Nat. Med.* **5**, 459–463.
46. Guerrero, R. B., Batts, K. P., Brandhagen, D. J., Germer, J. J., Perez, R. G., and Persing, D. H. (1997) Effects of formalin fixation and prolonged block storage on detection of hepatitis C virus RNA in liver tissue. *Diagn. Mol. Pathol.* **6**, 277–281.
47. Ohyama, H., Zhang, X., Kohno, Y., and Alevizos, M. (2000) Laser capture microdissection-generated target sample for high-density oligonucleotide array hybridization. *Biotechniques* **29**, 530–536.
48. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.
49. Maunsbach, A. B. (1966) The influence of different fixatives and fixation methods on the ultrastructure of rat kidney proximal tubule cells. II. Effects of varying osmolality, ionic strength, buffer system and fixative concentration of glutaraldehyde solutions. *J. Ultrastruct. Res.* **15**, 283–309.
50. Morales, A. R., Essendorf, H., Essendorf, E., Duboue, M. C., Vincek, V., and Nadji, M. (2002) Continuous-specimen-flow, high-throughput, 1-hour tissue processing. A system for rapid diagnostic tissue preparation. *Arch. Pathol. Lab. Med.* **126**, 583–590.



<http://www.springer.com/978-1-58829-107-3>

Pancreatic Cancer

Methods and Protocols

Su, G.H. (Ed.)

2005, XI, 352 p., Hardcover

ISBN: 978-1-58829-107-3

A product of Humana Press