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Laser Capture Microdissection

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Summary

Laser capture microdissection (LCM) is a technique for isolating pure cell populations from a heterogeneous tissue section or cytological preparation via direct visualization of the cells. This technique is applicable to molecular profiling of diseased and disease-free tissue, permitting correlation of cellular molecular signatures with specific cell populations. DNA, RNA, or protein analysis can be performed with the microdissected tissue by any method with adequate sensitivity. The principle components of LCM technology are (1) visualization of the cells of interest via microscopy, (2) transfer of laser energy to a thermolabile polymer with formation of a polymer–cell composite, and (3) removal of the cells of interest from the heterogeneous tissue section. LCM is compatible with a variety of tissue types, cellular staining methods, and tissue-preservation protocols that allow microdissection of fresh or archival specimens. LCM platforms are available as a manual system (PixCell; Arcturus Bioscience) or as an automated system (AutoPix™).

Key Words: Cancer; DNA; laser capture microdissection; molecular profiling; proteomics; protein; RNA; tissue heterogeneity.

1. Introduction

Laser capture microdissection (LCM) is a technique for isolating pure cell populations from a heterogeneous tissue section or cytological preparation via direct visualization of the cells. A common problem encountered by genomic and proteomic researchers in the analysis of tissue arises from the heterogeneous nature of the tissue. Molecular profiling of a pure cell population, which is reflective of the cell population's in vivo genomic and proteomic state, is essential for correlating molecular signatures in diseased and disease-free cells *(1–4)*. Direct microscopic visualization of the cells permits the selection of normal, premalignant, and malignant cells or disease and disease-free cells as

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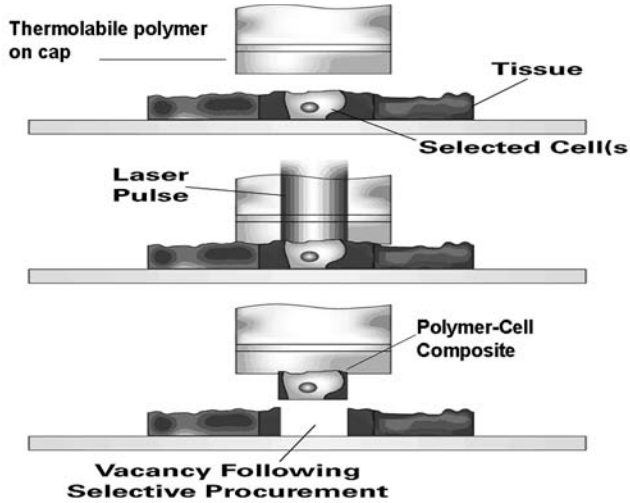

Fig. 1. Principles of LCM. A thermolabile polymer supported on an optical-quality plastic cap is positioned directly above the surface of a tissue section that is mounted on a glass microscope slide. A stationary, near-infrared laser is pulsed in the vicinity of the cells of interest. The polymer melts, or wets, in the vicinity of the laser pulse, forming a polymer–cell composite. Removal of the cap, away from the tissue surface, results in microdissection of the desired cells.

distinct cell populations from the heterogeneous tissue. Heterogeneous tissue might confuse molecular analysis because it is currently impossible to discern which cells contribute which cellular constituents to a given tissue lysate. LCM enables researchers to isolate specific cells of interest, without contamination from surrounding cells *(5–8)*.

Laser capture microdissection instruments as developed at the National Institutes of Health exist in manual and automated (robotic) platforms (Arcturus Bioscience, Inc., Mountain View, CA) *(9,10)*. The manual system, PixCell, and the automated system, AutoPix™ utilize identical principles for microdissection (*see* **Fig. 1**). The primary components of LCM technology are (1) visualization of the cells of interest via microscopy, (2) transfer of laser energy to a thermolabile polymer with formation of a polymer–cell composite, and (3) removal of the cells of interest from the heterogeneous tissue section.

A stationary near-infrared laser mounted in the optical axis of the microscope stage is used for melting, or wetting, a thermolabile polymer film (*see* **Note 1**). The polymer film is manufactured on the bottom surface of an optical-quality plastic support or cap. The cap acts as an optic for focusing the laser in the same plane as the tissue section. The polymer melts only in the vicinity

of the laser pulse, forming a polymer–cell composite. A dye incorporated into the polymer serves two purposes: (1) It absorbs laser energy, preventing damage to the cellular constituents, and (2) It aids in visualizing areas of melted polymer. Removal of the polymer from the tissue surface shears the embedded cells of interest away from the heterogeneous tissue section. Extraction buffers applied to the polymer film solubilize the cells, liberating the molecules of interest. The microdissected cells can be analyzed for DNA, RNA, or protein by any method with appropriate sensitivity *(6–9,11–15)*. Protein analysis of microdissected frozen-tissue sections will be used to illustrate LCM protocols.

2. Materials

2.1. Preparation of Tissue Sections or Cytospin Preps

1. Uncoated, precleaned glass microscope slides, 25 × 75 mm (A. Daigger & Co., Wheeling, IL).
2. Specimen for protein analysis of microdissected tissue: cytospin preps or frozen–tissue sections cut at 2–15 mm (*see* **Note 2**).
3. Specimen for DNA or RNA analysis of microdissected tissue: cytospin preps, frozen-tissue sections, ethanol- or formalin-fixed paraffin-embedded tissue sections cut at 2–15 mm (5–8 mm is optimal) (*see* **Note 2**).
4. Cryopreservation solution (OCT) (Sakura Finetek Corp., Torrance, CA).

2.2. H&E Staining of Tissue Sections

1. Mayer's Hematoxylin Solution (Sigma Diagnostics, St. Louis, MO). Hematoxylin is an inhalation and contact hazard. Wear gloves when handling.
2. Eosin Y Solution, alcoholic (Sigma Diagnostics). Eosin Y is flammable. Store away from heat, sparks, and open flames. Contact hazard; wear gloves when handling.
3. Scott's Tap Water Substitute Blueing Solution (Fisher Scientific, Pittsburgh, PA).
4. Ethanol gradient: 70% (v/v in purified H_2O), 95% and 100% ethanol. Prepare fresh ethanol solutions weekly, or sooner if staining more than 20 slides/wk or if the ambient humidity is greater than 40%.
5. Ethyl alcohol, absolute, 200 proof for molecular biology (Sigma-Aldrich, Milwaukee, WI). Ethanol is flammable. Store away from heat, sparks, and open flames. Do not ingest. Contact hazard; wear gloves when handling.
6. Purified water (Type I reagent-grade water).
7. Xylene (Mallinckrodt Baker, Inc., Phillipsburg, NJ). Xylene vapor is harmful or fatal; use with appropriate ventilation and discard in appropriate hazardous waste container. Xylene is flammable; store and use away from heat, sparks, and open flame. Contact hazard; wear gloves when handling.
8. Protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany).

2.3. Laser Capture Microdissection

1. PixCell II, PixCell IIe, or AutoPix laser capture microdissection system (Arcturus Engineering, Mountain View, CA).
2. CapSure™ Macro LCM Caps (Arcturus Bioscience) (*see* **Note 3**).
3. PrepStrip™ Tissue Preparation Strip (Arcturus Bioscience).
4. CapSure™ Cleanup pad (Arcturus Bioscience).
5. 500-µL Microcentrifuge tubes: Safe-Lock Eppendorf tubes (cat. no. 22 36 361-1; Brinkmann Instruments Inc., Westbury, NY) or MicroAmp™ 500 µL Thin-walled PCR Reaction Tubes (cat. no. 9N801-0611; Applied Biosystems, Foster City, CA).
6. Extraction buffer for cellular constituent of interest.

3. Methods

The following protocols illustrate (1) frozen-section sample preparation, (2) hematoxylin and eosin (H&E) tissue staining, (3) manual LCM for protein analysis, and (4) automated LCM. Alternative tissue preparation methods, such as ethanol or formalin fixation with paraffin embedding, are acceptable for DNA analysis of microdissected tissue ***(16)***.

3.1. Frozen-Tissue Sectioning

Frozen surgical biopsy material should be embedded directly in a cryopreservative solution or liquid nitrogen as soon as the specimen is procured. Prompt preservation of the sample limits protein degradation as a result of protease activity. The samples should be cut to 2–15 mm thickness (5–8 mm is optimal) on plain, uncharged, precleaned glass microscope slides. Position the tissue section near the center of the slide, avoiding the top and bottom third of the slide (*see* **Notes 2**, **4**, and **5**). Do not allow the tissue section to dry on the slide. Place the slide directly on dry ice or keep the slide in the cryostat at –20ºC or colder until the slides can be stored at –80ºC (*see* **Note 6**).

3.2. H&E Staining

Classic tissue-staining protocols allow visualization of the tissue or cells of interest with a standard inverted light microscope. Fixation of the tissue in 70% ethanol is followed by staining of the cellular constituents, with final dehydration in an ethanol gradient. Incorporation of protease inhibitors in the staining reagents, along with a microdissection session limited to 1 h, minimizes protein degradation during the staining process ***(17)*** (*see* **Note 7**). Most cellular staining protocols are compatible with LCM (*see* **Note 8**). Complete dehydration of the tissue is necessary for minimizing the upward adhesive forces between the tissue section and the slide (*see* **Note 9**). Fluorescent stains

are compatible with fluorescence-equipped PixCell systems and all AutoPix systems *(18)* (*see* **Note 10**).

3.2.1. H&E Staining for Frozen Tissue Sections

1. Remove slide from freezer and place on dry ice or directly into the 70% ethanol fixative bath.
2. Dip, or gently shake, the slide in each of the following solutions, for the time indicated. Blot the slide on absorbent paper in between each solution, preventing carryover from the previous solution.
3. 70% Ethanol fixative: 3–10 s.
4. Distilled (d)H_2O: 10 s.
5. Mayer's hematoxylin: 15 s.
6. dH_2O: 10 s.
7. Scott's tap water substitute: 10 s.
8. 70% Ethanol: 10 s.
9. Eosin Y (optional): 3–10 s.
10. 95% Ethanol: 10 s.
11. 95% Ethanol: 10 s.
12. 100% Ethanol: 30 s to 1 min.
13. 100% Ethanol: 30 s to 1 min.
14. Xylene: 30 s to 1 min.
15. Xylene: 30 s to 1 min.

3.2.2. H&E Staining for Formalin-Fixed Paraffin-Embedded Tissue Sections

Paraffin-embedded tissue sections must be deparaffinized prior to staining. Xylene acts as a solvent, removing the paraffin. Rehydration of the slide allows staining of the tissue elements.

1. Xylene: 5 min.
2. Xylene: 5 min.
3. 100% Ethanol: 30 s.
4. 95% Ethanol: 30 s.
5. 70% Ethanol: 30 s.
6. dH_2O: 10 s.
7. Mayer's hematoxylin: 15 s.
8. dH_2O: 10 s.
9. Scott's tap water substitute: 10 s.
10. 70% Ethanol: 10 s.
11. Eosin Y (optional): 3–10 s.
12. 95% Ethanol: 10 s.
13. 95% Ethanol: 10 s.
14. 100% Ethanol: 30 s to 1 min.
15. 100% Ethanol: 30 s to 1 min.

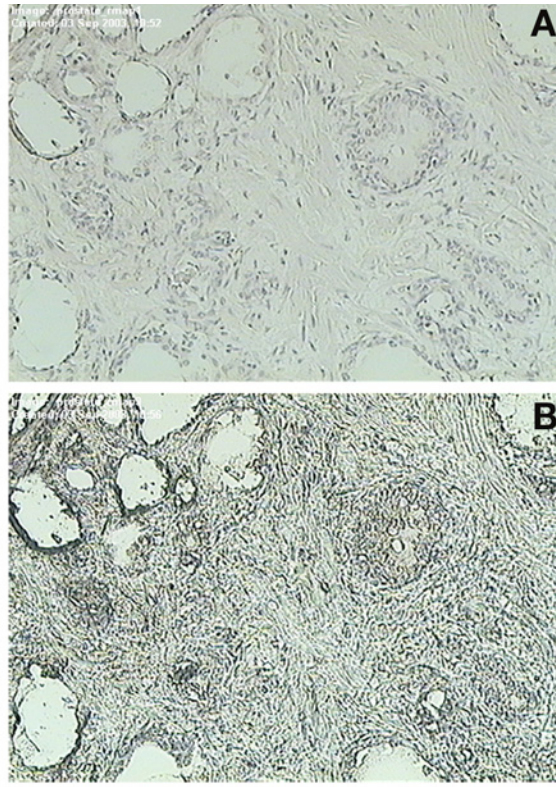

Fig. 2. Comparison of index-matched and non-index-matched tissue images. **(A)** Fluid, such as xylene, on the surface of the tissue acts as a refraction medium, providing an index-matched image of the tissue. As the slide dries **(B)**, the refractive index of the fluid is no longer present. The dry-slide image appears as shades of gray. This issue is overcome on the PixCell system by digitally saving the index-matched images of the tissue, or cells of interest, and using these images as a guide or map for microdissection.

16. Xylene: 30 s to 1 min.
17. Xylene: 30 s to 1 min.

3.3. Manual Laser Capture Microdissection (PixCell System)

There is no warm-up period required for the PixCell II/IIe. The instrument is ready for operation after turning on the power. Visualization of cellular morphological features and the tissue, in general, are achievable with image magnifications up to ×40 via optical and color digital imaging. Cover slips and mounting media are not compatible with microdissection. In addition, a lack of immersion fluids on any of the optics prevents refraction of light from the tissue image. Thus, the color and detail of a given tissue stain is lost as the stained slide dries (*see* **Fig. 2**). Manual LCM methods capitalize on the index refraction of a wet

tissue slide for visualizing and reviewing an index-matched image of the tissue (***19***,***20***). An index-matched image or images can be digitally saved and used as a guide, or scout, to locate the cells of interest after the stained slide dries.

3.3.1. Slide Preparation

Allow the stained slide to air-dry. Dust or debris on the slide can be removed by blotting the dried slide with a PrepStrip sample preparation strip.

3.3.2. PixCell Instrument Procedure for Microdissection

1. Load the CapSure cassette module with a CapSure cartridge.
   a. Remove the CapSure cassette module from the platform.
   b. Press in the locking pins on each end to hold the cassette in the load position.
   c. Slide a CapSure cartridge into the cassette until it stops. Two cartridges can be loaded onto the cassette module.
   d. After the cartridges are loaded, pull the locking pins out to lock the cartridges in place. Load the cassette module onto the PixCell II/IIe.
2. Access the LCM software program by double-clicking on the Arcturus software icon.
3. Enter your user name or select a name from the list. Click on "Acquire data."
4. Enter a study name or select a study name from the list. Click on "Select."
5. Enter the Slide # and Cap Lot #. If desired, notes concerning the slide or study can be entered as "Notes."
6. Click the checkbox for "Stamp images with name, date & time" if this information is to be imprinted on the images created during LCM. Click "Continue."
7. Move the joystick into the vertical position to ensure proper positioning of the cap in relation to the capture zone.
8. Place the microscope slide containing the prepared and stained specimen for microdissection on the stage.
9. Locate the cells of interest using either the oculars or the monitor. After the target area for dissection is in the viewing area, with the joystick still in the vertical position, press the "Vacuum" switch on the front of the Controller to activate the vacuum and hold the slide in place during microdissection.
10. The Live Video and PC screen displays the current image on the microscope. The images can be saved as you work by selecting the appropriate icon from the toolbar (*see* **Note 11**).

    Map image: low/high-power objective image of the general area or specific cells to be microdissected (*see* **Fig. 2**);
    Before image: intact tissue prior to microdissection;
    After image: tissue after microdissection;
    Cap image: microdissected tissue only.

11. Slide the CapSure cassette backward or forward so that a cap is sitting at the "Load" position. Swing the placement arm over the cap. While placing one hand over the counterbalance to prevent jarring of the cap and improper seating, lift the placement arm and place the cap onto the slide. This is the transfer position.

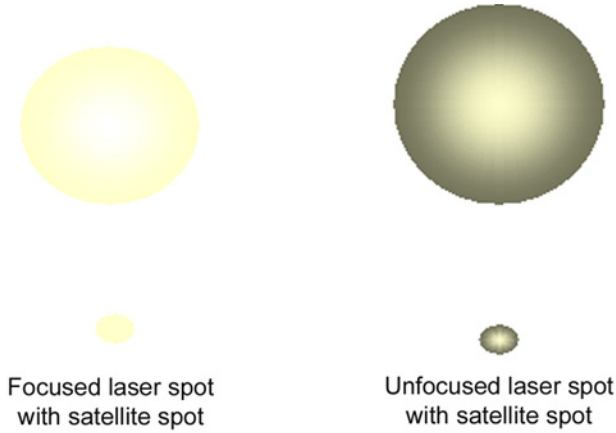

Fig. 3. Laser spot focus. A focused laser spot is essential for efficient microdissection. A focused laser appears as a bright, well-defined circle without a halo or corona. Unfocused laser spots appear as blurry spots with halos or coronas around a center bright spot. AutoPix instruments exhibit a satellite spot near the principle laser spot. The satellite spot is an optical phenomenon because of second-surface reflection of the laser beam as it propagates through the optical system. The satellite spot is a byproduct of the optical system but is helpful for determining the proper focus of the primary laser spot.

12. Enable the laser by turning the key switch located on the front of the controller and then press the "LASER ENABLE" button. Pressing this button will activate the target beam when the placement arm is in the transfer position.
13. Verify that the laser is in focus.
    a. Select the small spot size (7.5 µm), using the "Spot Size Adjust" lever found on the left side of the microscope.
    b. Rotate the objectives of the microscope until the 10x objective is in use.
    c. Using the joystick, move the slide under the laser so that target beam is located in an area without tissue.
    d. Reduce the intensity of the light through the optics until the field viewed on the monitor is almost dark and the target beam is easily viewed.
    e. Using the "Laser Focus Adjust" located just below the size adjustment lever, adjust the target beam until the beam reaches the point of sharpest intensity and most concentrated light with little or no "haloing" or coronas (*see* **Fig. 3**). The laser should now be focused for any of the three laser sizes and objectives. Select the laser spot size suitable for the microdissection and cell size. (*See* **Notes 12** and **13.**)
14. Press the red pendant button to fire a test laser pulse. Observe the wetted polymer after the laser is fired. Firing the laser pulse causes the polymer to melt in the vicinity of the laser pulse. There should be a distinct clear circle surrounded by a dark ring (*see* **Fig. 4**) (*see* **Note 14**).

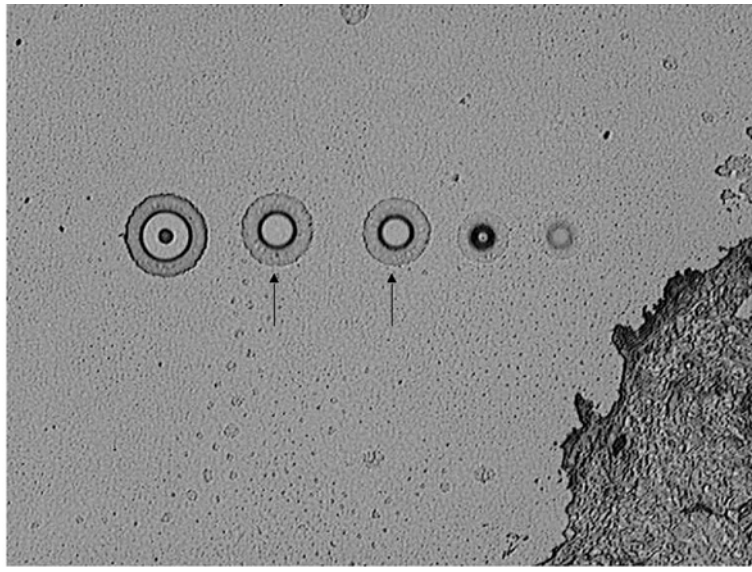

Fig. 4. Wetted polymer for effective microdissection. Adequate power, duration, proper tissue thickness, and cap placement on the tissue are parameters affecting the melting of the polymer. An ideal wetted polymer appears as a distinct dark ring with a clear center (arrows). The presence of a dark area in the center of the spot indicates that the power and/or duration settings are too high (leftmost spot). Inadequate power and duration result in failure of the laser to melt the polymer, creating a spot that appears as a gray, fuzzy circle (rightmost spot). Adequate power with inadequate duration might lead to spots with minute diameters, as shown by the spot second from the right.

15. Adjust the "Power" and "Duration" of the laser pulse with the up and down arrows on the front of the controller to obtain a melted polymer spot with a diameter similar in size to the selected laser spot size. Use the suggested ranges as a reference point. These settings can be adjusted to customize the melted polymer spot to the type and thickness of the tissue to be dissected. The suggested settings for the PixCell II system with a Macro cap are as follows:

Spot size	Power	Duration
Small, 7.5 µm	45 mW	750 µs
Medium, 15 µm	35 mW	1.5 ms
Large, 30 µm	25 mW	5.0 ms

16. Single-cell microdissection is possible by adjusting the power and duration settings such that a very narrow area of the polymer is melted with each laser pulse. Suggested settings for single-cell microdissection are approx 45 mW power and 650 µsec duration for PixCell II and Macro caps.

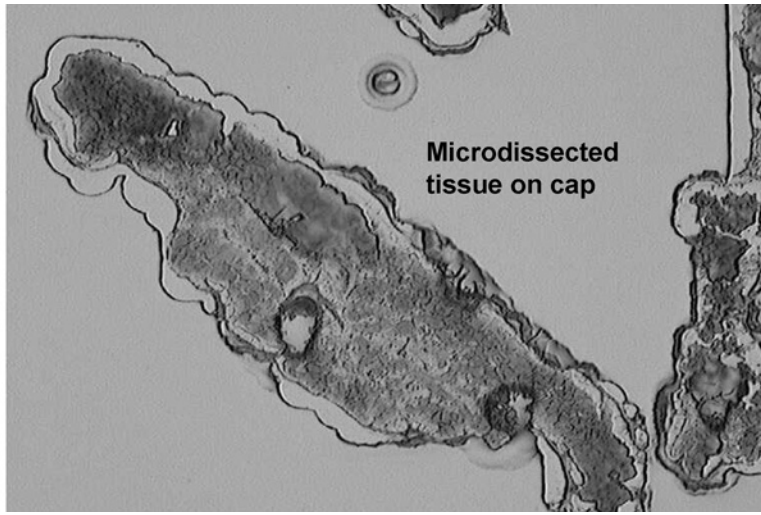

Fig. 5. Microdissected tissue embedded in the polymer cap. After microdissection, the polymer–cell composite can be visualized by placing the cap on the glass microscope slide in an area lacking tissue. The presence of stained cellular material inside the wetted margins of the polymer indicates effective microdissection.

17. To perform the microdissection, visually locate the cells of interest. Align the target beam directly over the cells of interest. Using the target beam to guide the dissection, press the pendant switch for single shots. For a rapid fire of pulses, press and hold the pendent switch. The laser pulse frequency interval can be adjusted on the controller by selecting "REPEAT" and then selecting the desired time between laser pulses.
18. After the desired number of cells has been collected on a cap, remove the cap by lifting it off the slide using the placement arm (*see* **Note 15**). Swing the placement arm away from the slide.
19. If desired, the microdissected material can be viewed by placing the cap on an area of the slide without tissue. Turn the vacuum off by depressing the "vacuum" button on the controller. Position the slide so the tissue is not in view on the monitor or through the oculars. Swing the placement arm, containing the cap, back onto the slide. Use the joystick to manipulate the cap above the objective. Observe the cap for microdissection of the desired cells and for debris and/or adhesion of nonspecific tissue to the polymer surface (*see* **Fig. 5**).
20. If viewing the cap reveals debris or nonspecific tissue adhesion, the said material can be removed by blotting the polymer surface with the CapSure Cleanup pad or an adhesive note.
21. Lift and rotate the cap arm until the cap is over the "cap removal site." Lower the arm and then rotate the arm back toward the slide. The cap will remain at the cap removal site. Blot the cap as described in **step 20** if necessary.

22. Insert the polymer end of the cap into the top of a 500-µL microcentrifuge tube. The sample is now ready for extraction of the desired components or the cap–tube assembly can be stored for extraction at a later date.
23. After all dissections are completed, the PixCell II/IIe should be put in shutdown by first pressing the "Laser Enable" button to disable the laser. Turn off the power to the PixCell II/IIe, the controller, and the video monitor.

3.3.3. Saving Images

Images saved during the microdissection can be saved on the computer hard drive.

1. Click the "Done" button on the image toolbar. Another slide can be microdissected, another study initiated, or the program terminated.
2. Click "Save Images" to save the images on the C drive of the PC. The images can be copied to a PC-formatted, 100-Mb zip disk as a .JPEG or .TIFF format after saving the image on the C drive. The AutoPix system is equipped with a CD-RW drive for file transfer and sharing.
3. Click on "Save Data."
4. Archived LCM images on the PixCell system are stored under the following file directory: C:\\LCMdata\user name\study name\date.

3.3.4. Storing Samples for Downstream Analysis

Microdissected cells for protein analysis can be stored at –80℃ prior to extraction. Microdissected cells for DNA analysis can be stored desiccated at room temperature up to 1 wk prior to extraction. Samples for RNA analysis should not be stored prior to extraction. Condensation in the microcentrifuge tube during storage can be a potential source of RNAse contamination.

3.4. Automated Laser Capture Microdissection (AutoPix System)

The AutoPix combines robotics and optical scanning software for automated microdissection of selected cells (*see* **Note 16**). The AutoPix incorporates imaging software for creating index-matched, stitched images of the tissue, permitting more accurate identification of cellular morphology during cell selection. The resolution of the stitched images is constant, but the area of the images changes with magnification, permitting precise areas of tissue to be annotated for microdissection.

Annotation software coupled with the index-matched image permits single-point dissection, line dissection or, polygon dissection (*see* **Fig. 6**; Color Plate 10, following p. 274). Algorithm-based, cell image recognition software on the AutoPix platform enhances the LCM technology. The algorithm is based on texture, morphology, size, color, and contrast of the tissue, permitting automated cell selection in addition to automated microdissection ***(21)***.

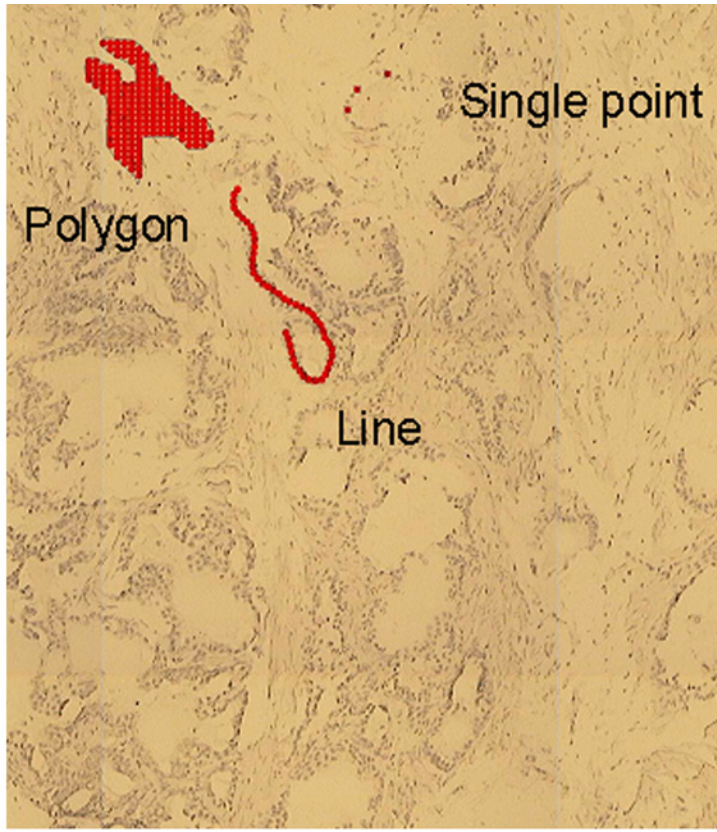

Fig. 6. Annotation of stitched images on AutoPix system. Annotation software permits single-point, line, or polygon area selection for microdissection on the index-matched stitched image. The ability to definitively select the cells of interest enhances the accuracy of microdissection. (*See* Color Plate 10, following p. 274.)

The AutoPix visualization system does not include oculars because of the enclosed system configuration. Instead, a PAL-format color camera permits visualization of the slide as a "roadmap image" for determining the target area of microdissection ***(21)***. The enhancements of the automated system include multiple-slide capacity (three slides), area quantitation of microdissected tissue, wetted polymer spot measurement, and cell recognition software.

4. Notes

1. The short laser pulse widths utilized, the low laser power levels required, the absorption of the laser pulse by the polymer and dye, and the long elapsed time between laser pulses combine to prevent the experimenter from depositing any

significant amount of heat at the tissue surface that might affect later laboratory analysis. The near-infrared laser diode has a maximum laser output of 100 mW.

2. Optimal tissue thickness for microdissection is 5–8 µm. Tissue sections less than 5 µm might not provide a full cell thickness, necessitating microdissection of more cells for a given assay. Tissue sections thicker than 8 µm might not microdissect completely, leaving integral cellular components adhering to the slide.
3. CapSure HS caps (Arcturus Bioscience) are often utilized in microdissection of tissue for RNA analysis. A 12-µm rail on the surface of the polymer prevents the polymer from touching the tissue except in the vicinity of the laser pulse. The HS caps are designed with an extraction device, allowing extraction buffer to contact the polymer within a centrally designated area. These features limit any potential RNA contamination from surrounding cells. CapSure HS caps can be used successfully for DNA or protein extraction. In contrast, CapSure Macro caps are placed in direct contact with the tissue and are not equipped with an extraction device. Any cellular material on the surface of the polymer of a Macro cap will be available for extraction.
4. The inverted light microscope in the PixCell platform utilizes a vacuum to immobilize the slide on the microscope stage. The size of the objective opening limits the microdissectable area on the microscope slide to the middle third of the slide. In contrast, the AutoPix stage has a capacity for one to three slides and the microdissectable area of the slide is approximately 19×45 mm.
5. Lung tissue or other tissue with a thin, open architecture can be cut on charged or silanized slides to prevent the tissue from nonspecifically adhering to the polymer during microdissection. In general, coated slides are not used for microdissection because of the increased adhesive forces between the tissue and the slide. Effective microdissection is a balance between three adhesive forces: (1) maximizing downward adhesive forces between the polymer and the tissue, (2) minimizing lateral adhesive forces between the cells, and (3) minimizing upward adhesive forces between the slide and the tissue.
6. Avoid repeated temperature fluctuations of the cut frozen sections. Store the frozen-section slides at –80℃ until the time of microdissection. Repeated fluctuations in temperature might cause the tissue to adhere more tightly to the slide, limiting the effectiveness of microdissection.
7. Protease and or phosphatase inhibitors can be added to compatible staining solutions *(**17**)*. Complete Protease Inhibitor Cocktail tablets are water soluble. For protease inhibitor addition to the 70% ethanol solution, dissolve the tablet in 15 mL of dH_2O, and then add 35 mL ethanol. Limiting the time from staining to completion of microdissection also ensures preservation of cellular constituents. Frozen-section samples for RNA and protein analysis should be stained and microdissected within 1 hr.
8. Examples of LCM compatible stains are H&E, methylene blue, Wright-Giemsa or toluidine blue. Eosin staining of the cytoplasm is not necessary for visualization of cells during microdissection. Minimal staining times, in whichever staining protocol is utilized, limit potential protein alterations as result of contact with

the staining reagents. Selection of tissue-staining protocols should be based on compatibility with the downstream analysis to be performed with the microdissected tissue.

9. Prolonging the 100% ethanol dehydration steps of the staining protocol to a maximum of 5 min could enhance tissue dehydration, maximizing microdissection efficiency. Skin tissue, cartilage, and samples prepared on charged slides might be difficult to microdissect. Additional slide or tissue treatments such as incorporation of glycerol slide coatings or modified staining protocols with glycerol might be required *(22)*. The following is one such approach for frozen-tissue sections as adapted from **ref.** *22*. Stain the slide in Mayer's hematoxylin for 30 s. Rinse in dH_2O for 15 s. Fix the slide in 70% and 95% ethanol solutions for 10 s each. Rinse in dH_2O for 10 s. Place the slide in Scott's Tap Water (Blueing) solution for 15 s. Dehydrate the slide in 70% ethanol for 2 min. Soak the slide in 3% glycerol in phosphate-buffered saline for 5–10 min. Dehydrate in two solutions of 100% ethanol: first solution for 10 s and second solution for 1 min. Clear the slide in two changes of xylene or xylene substitute for 1 min each. Allow the slide to air-dry before proceeding to microdissection.
10. PixCell instruments equipped with fluorescent modules incorporate mercury vapor lamps with blue, green, and red filter cubes. Additional filter cube positions are available for end-user modifications. Blue filter cubes use 455- to 495-nm excitation wavelengths, with emission greater than 510 nm. Green filter cubes use 503- to 547-nm excitation wavelengths, with emission greater than 565 nm. The red filter cube excitation wavelengths are 590–650 nm, with emission greater than 667 nm. These filter cubes can be used with immuno-LCM protocols *(18)*.
11. A drawback of the PixCell system is the inability to microdissect directly from an index-matched image of the tissue (*see* **Fig. 2**). Map images can be saved while the image is wet, providing a guide for microdissection *(19,20)*. This is not an issue with the AutoPix platform. Cells for microdissection are selected via annotation software directly from an index-matched, stitched image (*see* **Fig. 6**). Index-matched images can be obtained with either system by rewetting the tissue with a drop of xylene prior to microdissection. It is imperative that the slide be completely dry prior to cap placement for microdissection because xylene dissolves the polymer.
12. The laser should not be refocused when changing objectives or spot sizes. It is only necessary to focus the laser, with the small 7.5-µm spot size setting and the 10× objective, for each initial cap placement and any time the cap is repositioned on the tissue. Microdissection can be performed with any suitable laser spot size and a 4×, 10× or 20× objective.
13. Satellite laser spots are a phenomenon noted on the AutoPix platform (*see* **Fig. 3**). The satellite laser spot is a second-surface reflection of the laser as the laser beam propagates through the optics. The laser is reflected from the back (second) surface of a coated optic. The coating is required to allow the laser to change direction in the optical path. Imperfections in the coating allow a portion of the laser beam to pass through the coating and reflect back to the viewer from the second

optical surface. The power of the satellite spot is too low to melt the polymer and does not interfere with efficient microdissection. The satellite spot is a byproduct of the light amplification system and was not designed as a system component, but the satellite spot is helpful for accurately focusing the primary laser spot. If the satellite spot is in focus, the primary laser spot will be in focus.

14. The dark ring produced by pulsing the laser is a combination of migration of the dye and changes in the thickness of the polymer wall at the site of the laser pulse (*see* **Fig. 4**). These changes in the polymer permit visualization of the melted polymer. The black ring should be sharp in appearance with a clear center. This pattern indicates: (1) proper laser focusing, (2) adequate laser operation, and (3) acceptable performance of the CapSure polymer. A "fuzzy" ring could indicate improper focusing of the laser, uneven placement of the CapSure cap on the tissue, or inadequate power and/or duration of the laser pulse. The first step in troubleshooting a poorly wetted polymer spot is repositioning the cap on the tissue. Often, the cap is crooked or uneven in relation to the tissue. The second step in resolving poorly wetted polymer spots is refocusing the laser. The third action to correct poor polymer wetting is adjustment of the power and duration. Increase the laser power by approx 10 mW and the duration by 2.0 ms and fire another laser test pulse. Observe the wetted polymer for the appropriate appearance. If the above steps fail to resolve the problem, discard the cap and repeat the process with a fresh cap.
15. Assuming an average epithelial cell diameter of 7 µm and a 30-µm laser spot size, the operator can expect to collect, on average, five to six cells per laser pulse. Using this information, it is possible to estimate the number of cells captured based on the number of laser pulses counted during microdissection. The number of laser pulses is automatically counted on the toolbar on both the PixCell and AutoPix systems.
    a. 30-µm laser spot size: Number of pulses × 5 = total cells captured.
    b. 15-µm laser spot size: Number of pulses × 3 = total cells captured.
    c. 7.5-µm laser spot size: Number of pulses × 1 = total cells captured.
16. The AutoPix requires a warm-up period of 1 h prior to use if the instrument has been turned off. The instrument power can remain on for daily operation eliminating the need for a warm-up period.
17. The AutoPix is outfitted with a xenon lamp for fluorescence visualization, with similar filter cube configurations as the PixCell LCM system. The AutoPix is equipped with a high-sensitivity black-and-white camera in addition to the color camera.

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