

Optimized protocol for laser microdissection of living in-vitro cells



INTRODUCTION

Laser Capture Microdissection (LCM) is a proven technique for the isolation of pure cell populations for downstream molecular analysis. The combined use of UV laser cutting with LCM using the infrared (IR) laser allows for the rapid and precise isolation of larger numbers of cells, while maintaining cellular and nucleic acid integrity necessary for downstream analysis. In this note, it is shown that these established techniques can also be used for the isolation of living cells, avoiding other more laborious methods of cell selection, and providing opportunity for a wide range of research applications.

This application note describes a simple and effective protocol for the isolation of living adherent cells and the successful subsequent re-cultivation of homogeneous subpopulations. To demonstrate the success of this technique, two cell lines were used: TM3 (normal mouse testis) and SKBR3 (human breast cancer).

MATERIALS

- Laser Capture Microdissection Instrument (Laxco, Inc. Accuva Collect)
- PEN membrane frame slide (ThermoFisherCat. #LCM0521)
- CapSure®Macro LCM caps(ThermoFisher Cat. #LCM0211)
- Trypsin-EDTA(0.25%Trypsin,1mM EDTA, ThermoFisher Cat. #25200-056)
- Hanks' Balanced Salt Solution(ThermoFisher Cat. #14170-161)

APPLICATION NOTE #11

- Petri dish, sterile, 100mm diameter (Falcon Cat. #351005)
- Coverglass, 24x60mm, No.1 thickness (VWR Cat. #48393-106)
- RNase Away® (ThermoFisher Cat. #10328-011)
- 100% ethanol (VWRCat.#34172-00)
- Veritas Application Note
- CO2 incubator (MLS)
- Pipettor and sterile pipette tips (MLS)
- 2-well chamber slides (VWRCat.#62407-325)
- ND-1000UV/Vis Spectro photometer (Nano-Drop Technologies)
- Agilent 2100 Bio analyzer (Agilent Technologies Cat. #G2940CA)
- SKBR3 cell culture medium
- °McCoy's modified 5A media (ThermoFisher Cat. #16600-082)
- °10% Fetal Bovine Serum (FBS, ThermoFisher Cat. #16600-044)
- °1 mm Sodium Pyruvate (ThermoFisher Cat. #11360-070)
- °10 U Penicillin/10 µg Streptomycin (BioWhittaker Cat. #17-60ZE)
- TM3 cell culture medium
- °Dulbecco's Modified Eagle's Medium (ThermoFisher Cat. #30-2002)/Ham's F12 Medium (ThermoFisher Cat. #117650-054), 1:1
- °5% Horse Serum (ThermoFisher Cat. #16050-122)
- °2.5% Fetal Bovine Serum (ThermoFisher Cat. #16600-044)
- °10 U Penicillin/10 µg Streptomycin (BioWhittaker Cat. #17-60ZE)

METHODS

Specimen Preparation

Step 1. Thoroughly rinse the metal frame PEN membrane slide with 100% ethanol and air-dry prior to use. Keep slide in a sterile environment.

Note: Ensure the slide is completely dry prior to use.

Step 2. Trypsinize adherent cells from growth vessel (plate, flask) using standard protocol.

Step 3. Deactivate trypsin with media using standard protocol.

Step 4. Resuspend 1–2 mL of trypsinized cells in 10 mL of fresh media.

Step 5. Place a metal frame membrane slide with chamber facing up into a sterile petri dish. Transfer 1 mL of the cell suspension into the chamber of the frame membrane slide. If necessary, gently rock the slide in the petri dish to completely cover the chamber area with media.

Step 6. Place lid onto the petri dish and incubate using appropriate culturing conditions for the cells until desired cell confluency is achieved.

Note: Replace with fresh media as needed.

Laser Microdissection Slide Preparation

Step 1. Thoroughly clean the instrument and work area, including pipettors, pipette tip box, etc. with 100% ethanol and RNase- Away or RNase-Zap®.

Step 2. Thoroughly rinse a cover glass with 100% ethanol and air-dry prior to use. Keep cover glass in a sterile environment.

Note: Ensure the cover glass is completely dry prior to use.

Step 3. When cells have reached the desired confluency, remove the media from the chamber using a clean pipette tip.

Step 4. Add 950–1000 µL of fresh media to the chamber.

Step 5. Carefully place a cover glass over the chamber side of the frame slide to create a mini-environment for the cell culture, enabling extended survival and reducing the possibility of the cells drying out.
(See Figure 1.)

Note: Take care to reduce the amount of air bubbles formed when applying the cover slip.

Step 6. Using a Kimwipe,® carefully blot any excess media that has seeped outside the cover glass.

Step 7. Transport slide inside the petri dish to the LCM system.

Step 8. Remove the slide from the petri dish and use a Kimwipe soaked in 100% ethanol to clean the flat side of the frame slide. Dry the slide completely.

Note: Make sure not to rupture the membrane.

Step 9. Insert the frame slide with the chamber and cover glass facing down (flat side up) onto the LCM instrument and proceed to the laser microdissection session.
(See Figure 2.)

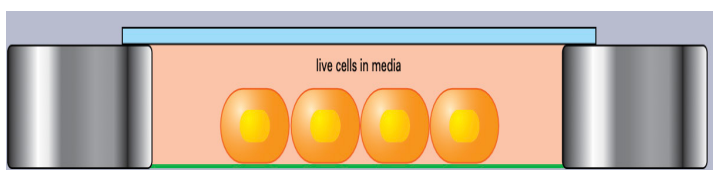
Laser Microdissection Protocol

The following settings were used for protocol validation and should be used as a guideline for the microdissection of live cells. Optimization of settings may be required depending on the individual cell preparation.

Important:

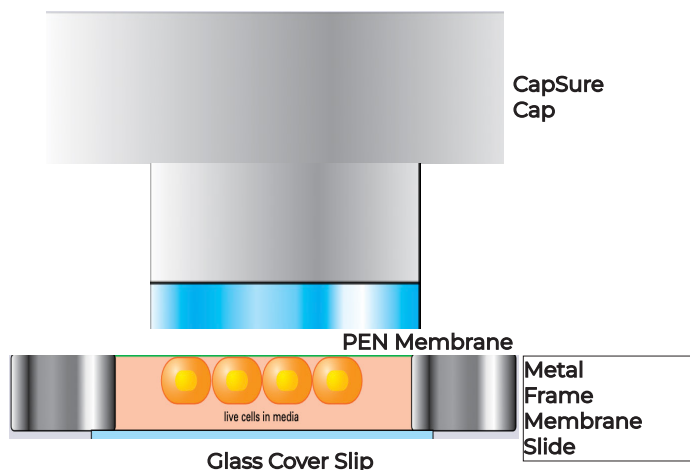
- Use CapSure Macro LCM caps.
- Perform cut and capture at 10x or 20x.
- Always capture first and then cut.
- Turn the visualizer off.

Laser Microdissection Cell Culture Chamber
(Figure 1)



Cells cultured in "well" of a metal frame membrane slide. The live cell culture is covered by a large cover glass prior to microdissection.

Inverted Laser Microdissection Cell Culture Chamber With Capsure Lcm Cap
(Figure 2)



The Capsure cap sits atop the inverted chamber in contact only with the PEN membrane during microdissection.

Step 1. Locate cells of interest to capture.

Step 2. Use the Cut Line feature to draw around cells.

Step 3. Use the Single Point capture feature to apply LCM spots that will fuse LCM membrane to PEN membrane.
Note: Apply adequate amount of LCM spots for the given region. Try to avoid placing spots directly onto areas containing cells.

Step 4. Place the CapSure Macro LCM cap onto the area of the slide containing cells of interest.

Step 5. Locate LCM laser and fire a test LCM shot. If necessary, adjust laser settings.

Note: Confirm that LCM film has made contact to PEN film. LCM spot will be dark.

Step 6. Locate UV cutting laser.

Step 7. Activate LCM laser first and then the UV cutting laser.

Step 8. Move Macro LCM cap to QC station.

Step 9. Confirm presence of cells on LCM cap. (See Figure 3.)

Step 10. Move stage to offload position.

Reculturing of captured live cells

Step 1. Remove Macro LCM cap from the offload station and invert. Place cap with isolated cells facing up into a clean petri dish.

Step 2. Pipette 50 μ L of Hank's solution onto the Macro LCM cap film surface. Pipette up and down 2–3 times, and dispose of the solution.

Step 3. Pipette 50 μ L of Trypsin-EDTA directly onto the captured cells on the cap and incubate for 5 minutes at room temperature. Cover with petri dish lid during this incubation.

Step 4. After incubation, pipette the Trypsin-EDTA up and down several times to ensure a single cell suspension, then transfer the cell suspension into a well of a sterile chamber slide (or alternate desired growth vessel) containing 1–2 mL of appropriate cell media.

Step 5. Place the chamber slide in the incubator under appropriate conditions. Wait 2–3 days for detection of cell growth. Monitor cell growth using standard culture technique, changing media as needed.

Step 6. Recultured cells may be used as desired for further experiments.

Results And Conclusions

Using two different cell lines, TM3 and SKBR3, crop circles were captured using the above techniques, each onto a separate CapSure LCM cap. Captured areas were designated as “Large” (2–3 mm diameter) or “Small” (500–700 μ m diameter) and contained varied numbers of cells. Subsequent to laser microdissection, some caps containing live cells were recultured following the above steps for protocol validation.

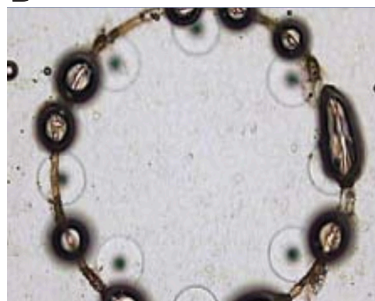
Alternately, microdissected cells were lysed and RNA was extracted to evaluate if there was any negative effect on the nucleic acid as a result of the microdissection protocol. These caps, containing captured live cells, were immediately placed into tubes containing PicoPure® RNA extraction buffer. The caps were placed into the extraction buffer prior to any reculture steps, immediately following removal of the cap from the LCM microdissection instrument. Total RNA was isolated following the standard PicoPure protocol, and eluted in 30 μ L of elution buffer.

Microdissection Of Living In-Vitro Cells Using Laser Microdissection (Figure 3)

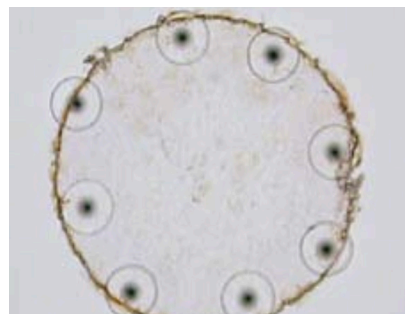
A



B



C



a: live cells in media before laser microdissection. B: Cell culture chamber after microdissection. C: microdissected live cells on the Capsure macro LCM cap.

The total RNA yields varied between replicates, as the cell counts within the captured areas were not monitored. However, the larger crop circles did result in higher yields than the smaller areas, as expected, and the RNA yield for each captured area, regardless of size, yielded sufficient RNA for downstream molecular analysis. The amount of RNA obtained from live cells subsequent to laser microdissection also indicates that the isolation protocol did not have a negative effect on the nucleic acids.

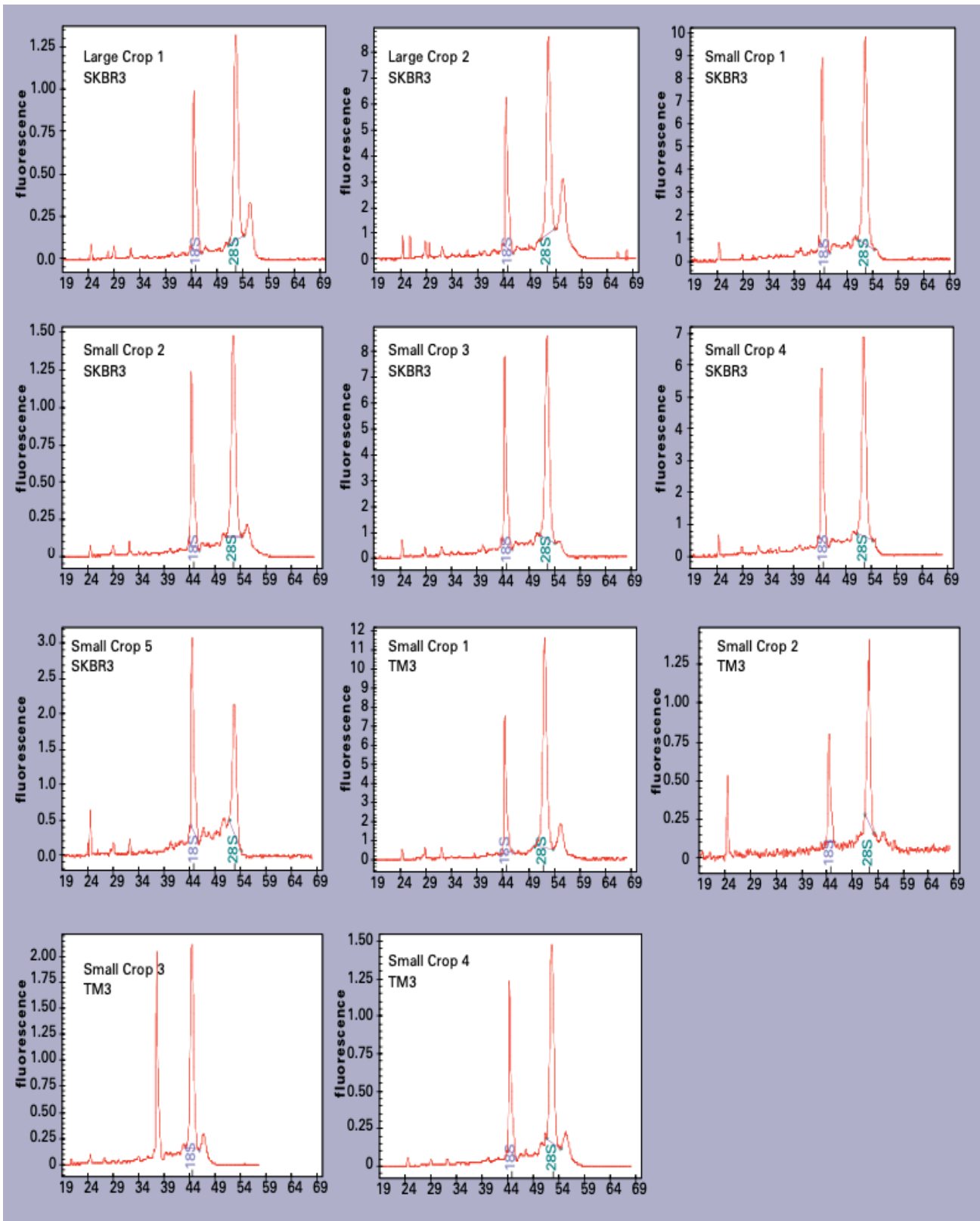
RNA profiles were obtained using the Agilent 2100 Bioanalyzer. (See Figure 4.) Clear 18S and 28S peaks are seen in each profile, further

Total RNA yield for each sample was assessed using the Nano Drop ND-1000. **(See Table 1.)**

Table 1. Total RNA Yield From Microdissected Live Cells				
Sample ID	Cell Type	RNA Conc. ng/μL	Total RNA Yield (ng)	Average Yield (ng)
Large Crop 1	SKBR-3	38.73	1161.90	964.65
Large Crop 2	SKBR-3	25.58	767.40	
Small Crop 1	SKBR-3	4.35	130.50	131.64
Small Crop 2	SKBR-3	6.73	201.90	
Small Crop 3	SKBR-3	3.77	113.10	
Small Crop 4	SKBR-3	4.44	133.20	
Small Crop 5	SKBR-3	2.65	79.50	
Small Crop 1	TM-3	4.34	130.20	184.74
Small Crop 2	TM-3	1.13	33.90	
Small Crop 3	TM-3	7.78	233.40	
Small Crop 4	TM-3	7.14	214.20	
Small Crop 5	TM-3	10.40	312.00	

Note: 1.5 μ L of each total RNA sample was measured using the NanoDrop ND-1000 UV/Vis spectrophotometer.

Bioanalyzer profiles of total RNA isolated from microdissected live cells (figure 4)



An amount of 1 μ l of each sample was loaded onto the Bioanalyzer and run per the manufacturer's protocol.

Note: Tm3 small Crop #5 is not present due to sample loading error.



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