Chapter 25

Laser Microdissection Microscopy: Application to Cell Culture

Ahlam Mustafa, Cathy Cenayko, Ragai R. Mitry, and Alberto Quaglia

Abstract

Laser microdissection (LMD) microscopy allows isolation of specific cell populations to target their molecular profile. There are several different types of LMD microscopes, but they are all based on the same principle. A laser beam is used to cut out cells or tissues of interest from a histological section, cytology preparations, or live cells from tissue cultures. Live cells can be isolated using LMD and processed for downstream molecular work. RNA, DNA, and protein isolation is possible from a small number of cells and the material is suitable for further real-time PCR, ELISA, Western Blotting, and protein microarray analysis.

Key words: Laser microdissection, HepG2, RNA, DNA, Protein and live cell microdissection

1. Introduction

Laser microdissection (LMD) is a well-established technique that allows the isolation of specific cell populations from tissue samples (1). In this chapter, we summarise the protocols we have developed for the isolation of live cells from tissue cultures using LMD and applications in molecular biology.

2. Materials

2.1. HepG2 Cell Line

Human hepatocellular carcinoma cell line (HepG2, Cat. No. 85011430; Sigma-Aldrich Ltd., Dorset, UK).

Ragai R. Mitry and Robin D. Hughes (eds.), *Human Cell Culture Protocols*, Methods in Molecular Biology, vol. 806,
DOI 10.1007/978-1-61779-367-7_25, © Springer Science+Business Media, LLC 2012

2.2. Chemicals and Solutions

The following is a list of chemicals and solutions used in tissue culture of HepG2 cells and further LMD, RNA extraction, and protein lysis.

1. RPMI 1640 with L-glutamine (Cat No. 21875; Invitrogen Ltd., Paisley, UK).
2. Foetal calf serum (FCS), heat inactivated (Cat. No. 10108-165; Invitrogen Ltd.).
3. Penicillin–streptomycin (Cat. No. 15140163; Invitrogen Ltd.).
4. Rnase away (Cat. No. 732-2351; VWR International, Leicestershire, UK).
5. TRIZOL® reagent (Cat. No. 15596-0026; Invitrogen Ltd.).
6. Chloroform (Cat. No. 2432-25 ml; Sigma–Aldrich Ltd.).
7. 2-Propanol (isopropyl alcohol, Cat. No. 19516 – 25 ml; Sigma–Aldrich Ltd.).
8. Ethanol (Cat. No. E7023 – 500 ml; Sigma–Aldrich Ltd.).
9. DEPC-treated Water (DEPC-H_2O, Cat. No. AM9915G; Invitrogen Ltd.).
10. 1× Phosphate-buffered saline (PBS) solution (Cat. No. 10010-05; Invitrogen Ltd.).
11. Aprotonin (Cat. No. A6279-10 ml; Sigma–Aldrich Ltd.).
12. Phenylmethylsulfonyl fluoride (PMSF, Cat. No. P7626; Sigma–Aldrich Ltd.).
13. Sodium orthovanadate (Cat. No. S-6508; Sigma–Aldrich Ltd.).
14. Igepal CA-630 (NP-40, Cat. No. 56741; Sigma–Aldrich Ltd.).
15. Deoxycholic acid “sodium salt” (Cat. No. D-5670; Sigma–Aldrich Ltd.).
16. Sodium dodecyl sulphate (SDS, Cat. No. 444464T; VWR International).
17. Sodium carbonate anhydrous (Na_2CO_3, Cat. No. 102404H; VWR International).
18. L(+)Tartaric acid “dipotassium salt” (Cat. No. T6897; Sigma–Aldrich Ltd.).
19. Sodium hydroxide pellets (NaOH, Cat. No. 28244.262; VWR International).
20. Cupric sulfate pentahydrate ($CuSO_4$, Cat. No. C-7631; Sigma–Aldrich Ltd.).
21. Folin and Ciocalteu’s solution (Cat. No. F9252 – 100 ml; Sigma–Aldrich Ltd.).
22. Bovine serum albumin (BSA) 10 mg/ml, (Cat. No. A2153; Sigma–Aldrich Ltd.).

2.3. Preparation of Solutions

1. The culture medium should be stored at 4–8°C and prepared by adding the following supplements to 500 ml of RPMI.
   (a) 50 ml FCS.
   (b) 5 ml penicillin–streptomycin.
2. Prepare RIPA buffer by adding the following to PBS, and store at room temperature (RT).
   (a) NP-40 (1% final concentration).
   (b) Sodium deoxycholate (0.5%).
   (c) SDS (0.1%).
3. Before using RIPA in preparing tissue lysate, freshly add the following:
   (a) 10 μl/ml PMSF.
   (b) 30 μl/ml aprotinin.
   (c) 10 μl/ml sodium orthovanadate.
4. Prepare alkaline copper reagent by dissolving the following into 80 ml of distilled water, and top the solution up to 100 ml with distilled water.
   (a) 10 g Na_2CO_3.
   (b) 100 mg tartaric acid.
   (c) 2 g NaOH.

   Dissolve 50 mg $CuSO_4$ in a few drops of distilled water and then add to the above mixture.
5. Dilute Folin and Ciocalteu's solution by adding 8 ml of distilled water to 0.5 ml of the stock (this has to be prepared freshly prior to immediate use).

2.4. Laser Microdissection Microscope and Accessories

1. Microscope Leica DM6000 B (Leica Microsystems Ltd., Knowlhill, Milton Keynes, UK).
2. Laser FTSS355-50 (Cat. No. 11501466; Leica Microsystems).
3. Camera DFC300 FX (Cat. No. 12730046; Leica Microsystems).
4. Operating System Leica Application Suite: Version 6.5.0.3104 (Leica Microsystems).
5. Scanning stage collector: 0.5 ml PCR tube (Cat. No. 11505228; Leica Microsystems).
6. Scanning stage collector: 8-well strip (Cat. No.11 505 230; Leica Microsystems).
7. Scanning stage Holder: Petri dish, ∅ 5 cm (Cat. No.11 505 227; Leica Microsystems).
8. 50 mm Petri dish with PEN membrane (Cat. No.11505172; Leica Microsystems).

9. 0.5 ml PCR tubes (Cat. No. 30124502; Leica Microsystems).
10. 8-Well Strips (Cat. No.11505240; Leica Microsystems).

2.5. Others

1. Refrigerated bench-top centrifuge for tubes and plates.
2. Water bath.
3. Suction pump.
4. Laminar flow cabinet.
5. Humidified incubator (37°C, 5% CO_2).
6. Maxwell®16 instrument (Cat. No. AS2000-SX; Promega UK, Southampton, UK).
7. Maxwell®16 FFPE Tissue LEV DNA Purification Kit (Cat. No. AS1130, Promega, UK).
8. Flat-bottom, 96-well plates with lids (Cat. No. 734-2097; VWR International).
9. Nanodrop® spectrophotometer (Labtech international, UK).
10. Plate reader to read 96-well plate at 650nm.

3. Methods

3.1. Cell Culture

1. Quickly thaw cryopreserved HepG2 cells stored in −140°C by gentle swirling of cryotube in a water bath at 37°C, taking care not to overheat the cells, i.e. cell suspension should remain cold after thawing.
2. In a laminar flow cabinet, transfer cells into a fresh 50-ml falcon® tube by passing them through a 70-µm sieve to remove clumps and clots. Slowly add medium (drop by drop) in a ratio of 10× the volume of the frozen Cells. (For 1 ml cells, add 10 ml medium).
3. Centrifuge solution to pellet cells at 450×*g*, 4 min, at RT. Discard supernatant. Re-suspend cells by tapping on the bottom of the tube, and then add 5 to 7 ml medium per 1 ml of cells available.
4. Estimate the cell count and viability using the standard Trypan blue exclusion technique (2).
5. Place 0.5×10^6 cells per Petri dish and top up with medium to total of 1.5 ml. Incubate cells at 37°C for 24–48 h.

3.2. Laser Microdissection

1. Switch on the Leica microscope electronics box, computer hard drive, and the laser.
2. Select PCR tube as collection device mode for samples to be used for further molecular work and eightfold holder with 8-well strip for re-cultivating LMD cells.

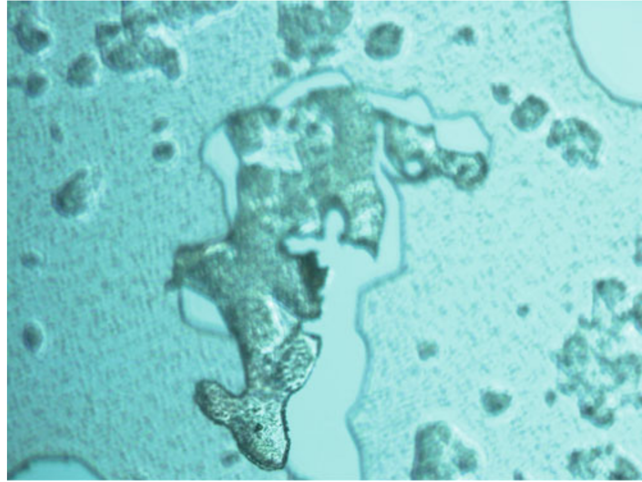

Fig. 1. HepG2 cell area falling into the PCR cap following LMD.

3. Insert the 50 mm Petri dish with PEN membrane and cultured cells into the sample holder.
4. Calibrate laser to the following parameters:
   (a) Power 42.
   (b) Speed 06.
   (c) Specimen Balance 13.
5. Select cutting area: Identify cells of interest. Select "Draw shape" options and trace around cells and cut using the laser (Fig. 1). Differential interference contrast may be used to clearly identify cells. The software is able to identify the area size being dissecting in μm^2.
6. Cells fall into the cap of a collection PCR tube or into one of the 8-well strip compartments for re-cultivating. Always check cells in the cap or the well to ensure proper localisation (Fig. 2).
7. Remove sample: It is advised to remove each PCR tube after each allocation of cells is reached; the 8-well strip can remain in the holder for longer (see Note 1).

3.3. Total RNA Extraction

Total RNA was extracted using TRIZOL® according to manufacturer's protocols with some modifications as below.

1. Clean all surfaces with RNase away.
2. Place 25 μl of TRIZOL® in the cap of a sterile 0.5 ml PCR tube prior to LMD. Following the collection of LMD cells, add a further 25 μl of TRIZOL® to the cap, and carefully close it.

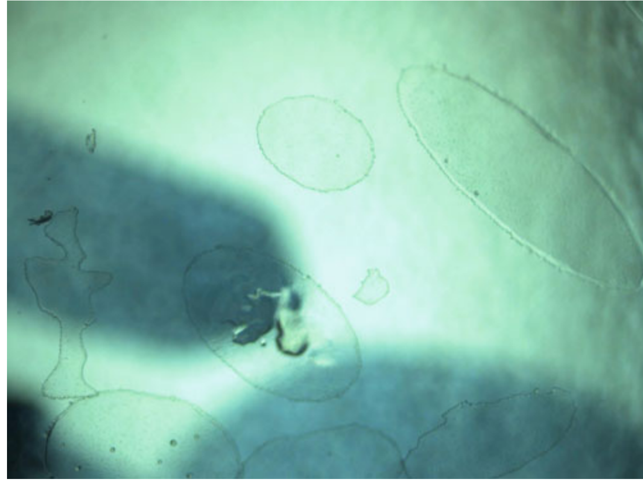

Fig. 2. Laser-microdissected HepG2 areas seen as oval shapes in the collecting PCR tube cap.

3. Allow LMD samples to lyse at RT for 5 min, followed by brief vortexing (see Note 2).
4. Add 10 µl of chloroform to each sample and shake vigorously for 15 s followed by incubation at RT for 3 min. Then, centrifuge at 12,000 × *g*, 4°C, for 10 min.
5. Transfer the aqueous layer carefully to a fresh sterile tube. Add 25 µl isopropyl alcohol per sample and mix well.
6. Incubate at RT for 10 min followed by centrifugation at 12,000 × *g*, 4°C, for 10 min.
7. Discard the supernatant, wash RNA pellet carefully with 60 µl of 75% ethanol, and centrifuge at 7,500 × *g*, 4°C, for 5 min.
8. Discard supernatant and leave pellet to air dry for 7 min in laminar flow cabinet. Dissolve each RNA pellet in 6.2 µl sterile DEPC–H_2O at 55°C for 7 min.
9. Read RNA concentration at 260 nm and assess quality (260/280 ratio) using the Nanodrop® spectrophotometer. It is possible to obtain an average of 79.7 ng/µl obtain from an area of 2.5×10^6 µm^2 of cells with an average 260/280 ratio of 1.7.
10. Extracted RNA is suitable for real-time PCR.

3.4. DNA Extraction

1. Place 25 µl of PBS in the cap of a sterile 0.5 ml PCR tube prior to LMD. Following sample collection, add a further 75 µl of PBS, close cap carefully, and spin tubes to ensure mixing (see Note 3).
2. Add two volumes of lysis buffer (provided in the DNA extraction kit – Subheading 2.5) to each sample and vortex briefly. Extract DNA using the Maxwell® instrument, DNA

purification Kit, according to manufacturer's protocol. It is possible to obtain an average of 57.3 ng/μl is possible to obtain from an area of 2.5×10^6 μm^2 of cells.

3. Extracted DNA is suitable for gene sequencing (see Note 4).

3.5. Tissue Lysate and Protein Concentration

Measuring protein concentration using Lowry technique (3) with some modification.

1. Place 25 μl of ice-cold RIPA buffer in the cap of sterile 0.5-ml PCR tube prior to dissection. After LMD is complete, add a further 25 μl of the lysis buffer to the cap and carefully close tubes.
2. Leave the LMD samples to lyse on ice for 30 min and then centrifuge at $15{,}000 \times g$, 4°C, for 20 min. Carefully collect supernatant (tissue lysate (see Note 5)).
3. Use tissue lysate to estimate protein concentration using Lowry technique with modifications as follows.
4. Prepare BSA standards using dilutions in Table 1.
5. Place 50 μl of the test sample or BSA standard per well. (Samples should be diluted using PBS to 1 in 3 volumes due to high concentration).
6. Add 50 μl of alkaline copper reagent to each well and mix content; incubate plate for 10 min at RT.
7. Add 200 μl of freshly prepared diluted Folin and Ciocalteu's reagent per well and mix content; incubate plate in RT for 15 min.
8. Read absorbance at 650 nm against blank. Plot the BSA standard curve and determine the protein concentration in each test sample. It is possible to obtain an average of 1,562 μg/ml from an area of 2.5×10^6 μm^2 of cells.
9. Tissue lysate is suitable for ELISA, Western Blotting, and protein microarray (see Note 6).

3.6. Isolating Cells for Re-Culturing

1. Place 50 μl of RPMI medium per well of the 8-well strip. After LMD of cells is complete, carefully collect all medium by pipetting and place into an allocated Petri dish.

Table 1
BSA standard curve

	Volume (μl)					
PBS	500	495	490	485	480	475
BSA	0	5	10	15	20	25
Final concentration (μg/ml)	0	100	200	300	400	500

2. Add an extra 1.5 ml of RPMI medium and incubate at 37°C for 24 h. Specific cells could be isolated using the same technique from a heterogeneous sample.

4. Notes

1. LMD cell areas are minute in size; to ensure full lysis in all protocols, keep microfuge tube inverted during the incubation period. Then, turn right side up and centrifuge to collect lysate at the bottom of the tube.
2. Samples may be frozen at –80°C for later analysis at two points (following the completion of steps 2 and 4) in the RNA extraction protocol.
3. In the DNA extraction protocol, volume of PBS used should not exceed 100 μl.
4. Methods of RNA, DNA, and protein extraction used in this chapter are not exclusive; other techniques may be applied with careful optimisation to render the procedure applicable to the small number of cells being analysed.
5. In the tissue lysis protocol, if the tissue lysate is not being processed immediately, add an extra 3 μl of PMSF per sample should be added for every 30 min of delay following the completion of step 2.
6. To use the protein lysate for protein microarray, exclude NP-40 and SDS from RIPA buffer and increase the cell areas being dissected by two- and threefold of minimum recommended in the protocol.

References

1. Espina V, Wulfkuhle JD, Calvert VS, VanMeter A, Zhou W, Coukos G, et al. Laser-capture microdissection. Nat Protoc 2006;1(2):586–603.
2. Freshney R. Culture of Animal Cells. New York, NY: Wiley-Liss; 2000.
3. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951 Nov;193(1):265–75.