CHAPTER 7

Capturing a Single Cell

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7.1 Introduction

The development of technologies for whole genome analysis of the transcriptome and proteome has allowed spectacular advancement in numerous biological fields. However, in many cases, the biological significance of results is tightly associated with the cellular composition of analyzed tissue. For example, genomic and proteomic analysis in the context of cancer investigation is susceptible to contamination by non-neoplastic cells (as inflammatory cells and vascular cells) which can mask tumor cell specific alterations. The degree of masking will depend on the percentage of tumor cells versus non-tumor cells located in the tissue analyzed.[1,2] The same problem of signal dilution is observed in all heterogeneous tissue and more particularly in complex tissue such as brain.

The development of technologies allowing the isolation of cells from heterogeneous tissue and methods to amplify small quantity of nucleic acids

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(DNA and RNA) answers this problem.[3] In this chapter, we will review these methods.

7.2 Overview of Cell Sorting Technologies

A first batch of approaches available to concentrate and purify cells of interest is to use cell sorting techniques such as density gradients,[4] fluorescence-activated cell sorting,[5] antibody-labeled magnetic beads.[6,7]

- The *density gradients* procedure is based on the possibility of producing a linear gradient density with different media as sucrose, metrizamide, Ficoll™ and Percoll and to separate cells on the basis of their density following centrifugation or sedimentation. This procedure is extensively used to separate monocytes from whole blood.
- *Fluorescence-activated cell sorting* is a specialized type of flow cytometry. It provides a method for isolating cell populations upon specific fluorescent labeling of cells of interest. The procedure uses several steps: (1) fluorescent labeling of intact cells, (2) separation of cells in individual liquid droplets, (3) measuring the fluorescence with a specific laser, (4) placement of electric charges in fluorescence-positive cells, and (5) separation of charged cells in an electric field.
- The method using *antibody-labeled magnetic beads* is based on the property of uniform polystyrene spherical beads to be magnetizable. The attachment of target-specific antibodies to the surface of the beads allows the capture and isolation of intact cells directly from a complex suspension of cells. This procedure is accomplished without intervention of column and centrifugation steps. Positive or negative cell isolation can be performed regardless of the availability of the antibody able to recognize specific cell surface markers. This technology is particularly used to isolate CD34 + stem cells.

These methods need the creation of a suspension of individual cells, which could be rarely applicable for solid tissue without disturbing cell phenotypes. Consequently, a chemical cell-dissociation step such as trypsinization is applied. The other approaches developed allow selective isolation of cells from their non-disturbed biological environment. These techniques – ranging from the lowest to the highest degree of resolution – include manual microdissection with a razor blade or needle attached to a micromanipulator,[8] and more recently technologies based on laser microdissection under microscopy cell visualization. The different technologies are presented in Table 7.1.

7.3 Laser Capture Microdissection Technologies

Laser capture microdissection (LCM) appeared in the 1970s but really became available during the mid 1990s with the work by the Emmert-Buck team at the

Table 7.1 Cell isolation methodologies.

Methodology	*Principle*	*Applications*
Density gradients	Cell sorting using media forming a gradient	Cell suspension
Fluorescence activated cell sorting (FACS)	Cell sorting using immunolabeling	Cell suspension
Magnetic beads (Dynabeads, Dynal Biotech) (www.invitrogen.com)	Cell isolation with magnetic beads coated with an antibody	Cell suspension
Needle attached to a micromanipulator	Mechanical isolation	Frozen and fixed paraffin-embedded section
Laser microdissection	Cell cutting or capture	Frozen and fixed paraffin-embedded section
	Mechanical cell recovery	Living cell culture, smears, cytospin

National Institutes of Health, Bethesda, USA.[9] There are two general classes of laser microdissection systems: infrared (IR) capture systems and ultraviolet (UV) cutting systems. The fundamental features of the laser microdissection process are: (1) visualization of the cells via microscopy, (2) transfer of laser energy to a thermoplastic film (IR system) or cutting surrounding a selected area (UV system), and (3) removal of the cells of interest from the heterogeneous tissue section by different procedures. The different characteristics are summarized in Table 7.2.

7.3.1 Infrared Laser Capture Systems

LCM technology (the Arcturus system, Figure 7.1) uses a low-power infrared laser to melt a special thermoplastic film over the cell(s) of interest (Figure 7.2). CapSure® HS or CapSure® Macro Caps, which are coated with this thermoplastic film and are especially developed for this technology, are placed on the tissue section or cytology sample. The PixCell® IIe LCM instrument is then used to direct the laser through the cap to melt the film onto the cells of interest. In addition, the CapSure LCM Caps work with ExtracSure™ devices that minimize the dilution of biological molecules extracted from captured cells. This optimizes the recovery of these molecules for downstream molecular analysis.

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:

- Absorption of laser energy, preventing damage to the cellular constituent
- Visualization of melted polymer areas

The combination of (1) the short laser pulse duration used, (2) a low laser power level, (3) absorption of the laser pulse by the polymer, and (4) the long

Table 7.2 Features of the equipment from different manufacturers.

System	*Laser*	*Resolution of dissection*	*Cell collection process*	*Specimen source*
Arcturus Pixcell II (www.moleculardevices.com)	IR (810 nm)	5–7.5 μm to 30 μm	Plastic melting and mechanical removal	Frozen and FFPE tissues, smears, cytospin
Biorad Clonis (www.microscopy.bio-rad.com)	IR (780 nm)	2 μm	Capture on a non-toxic absorbing film	Living cells and tissues
PALM (www.zeis.com)	UVa (337 nm)	0.5–1 μm	Pressure catapulting system	Frozen and FFPE tissue, smears, cytospin, living cells, chromosome spreads
Leica system (LMD 6000) (www.leica-microsystem.com)	UV (355 nm)	4–5 μm	Gravity	Frozen and FFPE tissue, smears, cytospin, living cells, chromosome spreads
Molecular Machines and Industries (MMI) (www.molecular-machines.com)	UV (350 nm)	1 μm	Adhesive Eppendorf cap	Frozen and FFPE tissue, smears, cytospin, living cells
Veritas, XT (Arcturus) (www.moleculardevices.com)	UV/IR (810/355 nm)	5/0.2 μm	Adhesive collection	Frozen and FFPE tissue, smears, cytospin, living cells, chromosome spreads

FFPE, formalin-fixed paraffin-embedded.

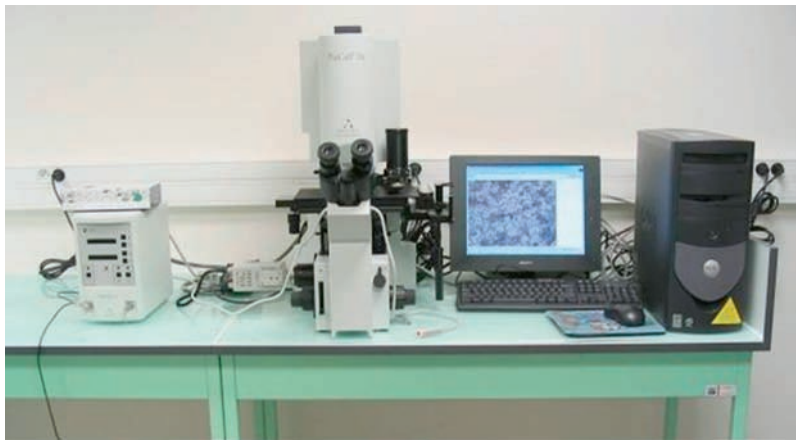

Figure 7.1 Laser capture microdissection (LCM) apparatus (Pixcell II from Arcturus).

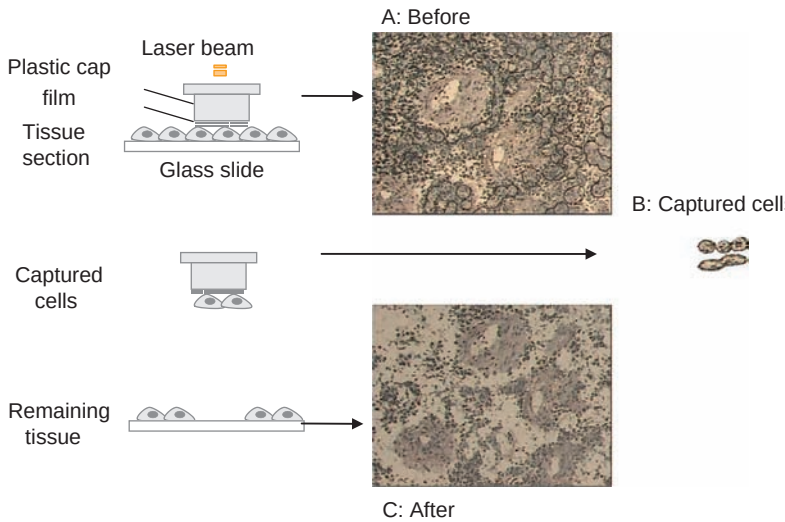

Figure 7.2 The principle of laser capture microdissection (LCM).

time between laser pulses, prevent any significant amount of heat deposition at the tissue surface that might affect molecular analysis.

Up to 3000–5000 cells can be isolated onto a single cap. Once all cells of interest have been captured, the caps are mechanically removed from the slide and unwanted cells remain attached to the microscopic slide. The power of the laser and its diameter can both be adjusted independently to adapt for the dissection of different kinds of tissues. After visual control of the cap, adhered target cells are subsequently lysed and DNA/RNA or protein extracted using extraction and purification methods with adequate sensitivity.

An automated version is available (AutoPix, Veritas and XT from Arcturus).[10] The Biorad clonis platform is dedicated to work on living cells.

7.3.2 Ultraviolet Cutting Systems

The shorter pulse duration in combination with the optimal optical transmission at 337–355 nm enables fast and highly precise cutting. However, these systems require special slides for the work. Tissue sections are first transferred onto a microscope slide covered by a thin polyethylene membrane. Single cells or group of cells are excised by circumcision with a high-energy focused laser beam. The laser cuts a beam spot size of less than 1 μm in diameter. For a UV system, unlike an IR system, a precise cutting line depends on the objective magnification. With LPC^{pat} technology (PALM),[11] the high photonic pressure force of the focused laser beam ejects selected sample from the object plane and catapults it directly into the cap of a microcentrifuge tube.[12]

UV cutting systems are particularly useful for the microdissection of tissue sections up to 200 μm thick, such as sections of plant tissue.

A potential limitation of the UV laser systems is the putative UV-induced damage in the final cell population. The UV system may be used to ablate unwanted tissue. Laser microbeam microdissection systems use a much thinner laser beam diameter in contrast to the IR-LCM and enable clear cut separation from the neighboring tissue.

The Molecular Machines and Industries (MMI) CellCut and SmartCut systems operate in the same way as the PALM. The Leica system uses the same cutting procedure but target cells are collected by placing an adhesive cap (of a microcentrifuge tube) onto the cut area.

The ArcturusXT™ and Veritas systems are a unique microdissection instruments that combine laser capture microdissection (LCM) and ultraviolet (UV) laser cutting in one platform.

7.4 Protocols Before Laser Microdissection (Tissue Sampling and Preparation)

To obtain relevant results from tissue isolated cells, the procedure involves the optimization of critical preparation steps:[13] (1) maintaining the tissue morphology to allow good identification of the cells of interest; and (2) maintain the integrity and a high yield for DNA/RNA and protein recovery after microdissection. These two critical steps are largely dependent on the tissue preparation (fresh frozen (FF) tissue and formalin-fixed paraffin-embedded (FFPE) tissue).

It was found that the main factors influencing tissue morphology, LCM capture success and acid nucleic/protein integrity are: (1) slide temperature for collecting tissue, (2) method and temperature of fixation, and (3) temperature and nature of staining and dehydration solution.

For the LCM system, dehydration steps are essential for capture success. The dehydration allows a decrease of the RNase or protease activity during microdissection.

7.4.1 Dissection from Fresh Frozen Tissue

Frozen sections are highly recommended to maximize quantity and quality of RNA/DNA and protein recovery.[14] In Figure 7.3 are presented two

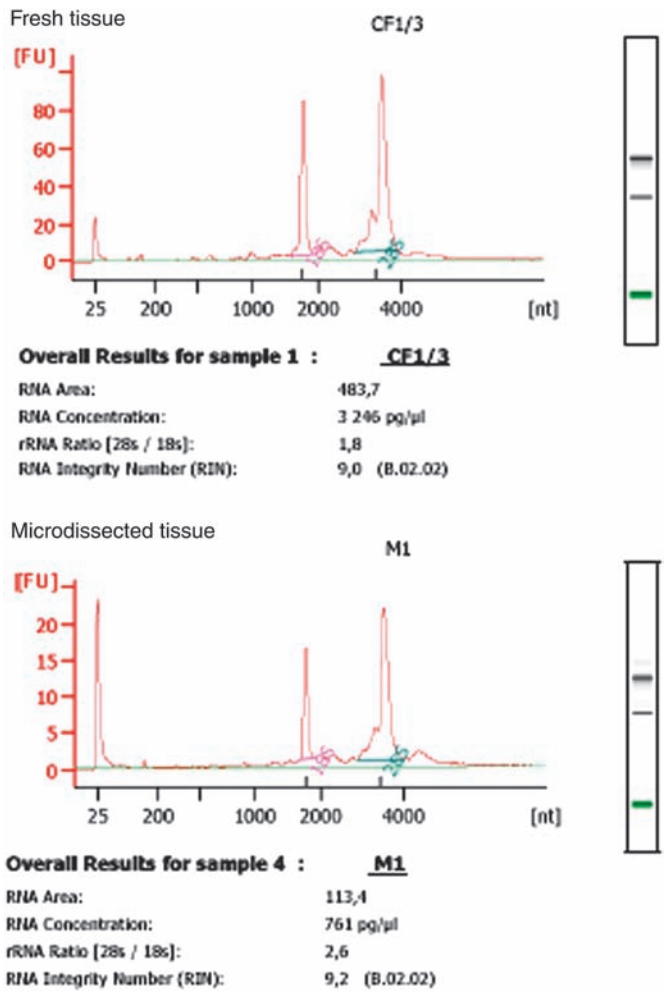

Figure 7.3 Comparison of RNA quality. Total RNA extracted from fresh tissues or the same microdissected tissue is analyzed using a 2100 Bioanalyzer (Agilent). The RNA integrity number (RIN) proves the good preservation of RNA quality during the whole procedure of IR microdissection (data from our group).

electropherograms (Bioanalyser 2100, Agilent) showing the very similar quality of two RNA preparations coming from fresh tissue or microdissected cells from the same tissue (data from our group). Different cryo-protection methods facilitate cutting frozen sections without freezing artifact. Optimal Cutting Temperature (OCT™) is a useful widely used product to preserve tissue morphology but this compound can inhibit polymerase chain reaction.[15]

However, in frozen section it is often difficult to recognize histological details after routine staining, such as with hematoxylin&eosin (H&E), because in this case laser microdissection requires desiccated sections without cover slips. To circumvent this limitation, specialized staining methods have been developed for distinguishing cells of interest from surrounding the stroma, including Nissl's stain (NS), immunofluorescence (IF), and immunohistochemistry (IHC). In most cases, cresyl violet staining will be sufficient for cell identification, a basic dye that stains negatively charged nucleic acids in the nucleus of a cell with a dark blue color. It also stains the rough endoplasmic reticulum of neurons and preserves RNA/DNA and proteins.

In the case of the tissue morphology damages, cresyl violet might not clearly show cell distribution. In such cases, the acridine orange stain may provide better contrast between clusters of cells.

The stain used before microdissection has to be compatible with a good preservation of molecular targets and must be checked in pilot experiments.

7.4.2 Dissection from Formalin-fixed Paraffin-embedded Tissue

In clinical research, it is often the case that morphology of frozen tissue is not sufficient for complete identification of cells of interest and the use of formalin-fixed paraffin-embedded tissues section is preferred. Formalin fixation operates by creating extensive cross-links within and between proteins. Disruption of the cross-links produces peptide and protein fragments rather than intact proteins. Despite the development of new technologies for reversing cross-linking, the yield and quality of protein/RNA/DNA remain low.[16,17] Different reversible cross-linkers, such as dithiobis(succinimidyl) propionate (DSP), have been developed and used successfully.[18] While the results of some studies are discouraging, archived FFPE samples have been successfully used in some cases to identify prognostic and diagnostic gene signatures for numerous diseases.[19,20] Paraffin-embedded sections can be used with conventional staining techniques, including immunostaining.

7.4.3 Immuno Laser Capture Microdissection

Immuno-LCM, which involves immunohistochemical staining of tissue before laser microdissection, enhances the ability to identify cells of interest in complex tissue by combining morphology and immunophenotype. Immunostaining protocols are available for frozen tissue or FFPE tissue. In this strategy, the optimal immuno-LCM procedure, in which RNA is not degraded during the

staining process, requires the use of high-affinity antibodies at high concentration with a short overall staining period.[21,22] It is worth mentioning that DNA *in situ* hybridization has also been successfully combined with laser microdissection to analyze cells based on their genotype.[23]

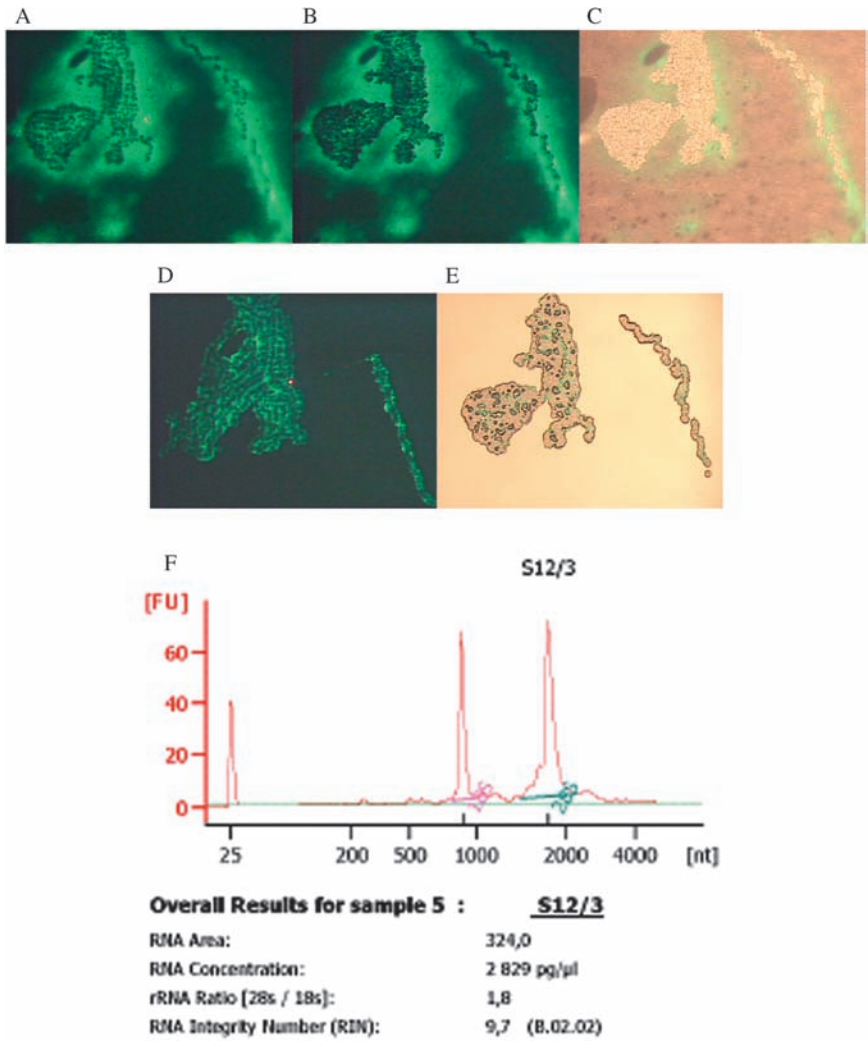

Figure 7.4 Laser capture microdissection of fluorescent cells. Laser microdissection has been performed on rat brain expressing GFP-siRNA directed against the Huntington gene. Microdissection is performed using Pixcell II from Arcturus under UV exposure. (A) Before capture; (B and C) after capture; (D and E) isolated cell on caps; (F) quality of total RNA obtained from microdissected cells.

7.4.4 Other Cell-labeling Methods

Other strategies can be used to avoid pre-treatment of cells which could lead to nucleic acid and protein degradation. These methods include: (1) the pre-labeling of cells *in vivo* in animal models by injection of a fluoro-gold label;[24] (2) the use of lectin probes for labeling brain microvessels; [25] and (3) the use of transgenic animals expressing the fluorescent transgene as green fluorescent protein (GFP) in specific cells is easily detectable under UV exposure.[26] An example of this last application achieved in our platform[27] is presented in Figure 7.4.

7.5 Conclusion

The development of LMD, which started in the 1990s, has made it possible to isolate defined cells from a heterogeneous cell population without contamination of unwanted cells.

Simultaneously, development of wide genome analysis technologies (genomic, transcriptomic, and proteomic) allow, ''in theory'', the single cell machinery to be deciphered. It may be argued that examining the expression profile of the complete cellular microenvironment of disease state (for example, in oncology) is more representative of the ongoing progress than the separation of individual cells. However, previous studies clearly showed that analysis of bulk tissue (tumor) gives few relevant data in comparison with the huge data collection and sample analysis (particularly for transcriptomic analysis). Studies that gradually break down the complex interactions that exist *in vivo* between neighboring cell types would greatly facilitate our understanding of normal and disease states.

All microdissection technologies are fundamentally different in terms of their physical principles and handling, and they have their limitations. Only the specific application can determine the most appropriate technology to use.

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