Laser microdissection of cellular compartments for expression analyses in cancer models

The lasermicrodissection of cellular compartments for expression analyses in cancer models

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Running Head: LMD for cellular compartment specific analysis

Abstract

Cancer tissues are composed of various cell types including cancer cells, cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs) and endothelial cells. These surrounding stromal cells form the tumor microenvironment, partly through the inflammatory response, which plays an important role in the development and malignant progression of cancer. It is therefore important to examine the expression profiles and protein modifications of each cellular component independently to decipher the interaction between the tumor cells and the microenvironment. We herein describe a protocol for laser microdissection, which allows for the individual cellular compartments to be collected separately. This will allow us to perform real-time RT-PCRs and microarray analyses of specific cell types in tumor tissues.

Key words: Laser microdissection, cancer model, frozen section, paraffin-embedded section, RNA extraction, DNA extraction

1. Introduction

It has been established that the tumor microenvironment, which consists of bone marrow-derived inflammatory cells and activated residential cells, supports cancer development [1]. CAFs and TAMs are major components of the tumor microenvironment, and play important roles, not only in the early stage of tumorigenesis, but also in the process of malignant progression, including metastasis and relapse [2, 3]. Recent studies have also indicated that innate immune responses through Toll-like receptors (TLRs) are activated both in the immune cells and in cancer cells, and that TLR signaling in both types of cells is essential for tumorigenesis through the activation of independent pathways [4, 5]. Moreover, there is heterogeneity within the same tumor tissue; for example, EMT cells or cancer stem cells may be present at the invasion front [6, 7]. Accordingly, it is important to examine the expression in different cellular compartments within tumor tissues by sampling the respective cell types separately, without contamination of other types of cells.

Previously, *in situ* hybridization (ISH) of specific mRNA was the standard method for determining the types of cells expressing specific genes. However, it was difficult to estimate the expression levels by ISH. In contrast, the collection of specific cells under a microscope by laser microdissection (LMD) allows us to prepare samples for both a real-time RT-PCR and a transcriptome analysis of mRNA and microRNA (miRNA), or next generation DNA

sequencing. Recent studies that have applied the LMD technique have revealed useful information about the heterogeneity of tumor tissues [8, 9]. In our laboratory, we have examined the tumor cell-specific expression of inflammation-related genes and miRNAs in gastric tumors [10] and the induction of tumor suppressor-related genes in the invasion front of intestinal tumors using LMD (unpublished). We herein describe the method that is routinely used in our laboratory to collect specific cellular compartments of tumor tissues from mouse gastric and intestinal tumors by LMD for use in real-time RT-PCRs, and miRNA microarray and genomic DNA analyses.

2. Materials

For the preparation of all solutions, use ultrapure water (prepared by purifying deionized water). Store all reagents at room temperature unless indicated otherwise. Diligently follow all waste disposal regulations when disposing of waste materials. It is recommended that commercial extraction kits be used for the extraction of RNA, miRNA, or DNA from small amounts of microdissected tissue.

2.1. Frozen sections and staining

1. Phosphate buffered saline (PBS) pH 7.4: Dilute 10 X PBS Buffer with ultrapure water.
2. Aluminum foil cups for embedding tissues (**Fig. 1**, see **Note 1**).
3. Tissue-Tek O.C.T. compound for embedding tissues (Sakura Finetek, USA).
4. LMD membrane slides covered with PEN-membrane 2.0 μm (Leica, No. 11505158), pretreated for RNase free before use (see **Note 2**).
5. Fixation solution, 19:1 mixture of ethanol:acetic acid mixture (see **Note 3**).
6. 0.05% Toluidine blue solution (pH 7.0) for staining frozen sections.

2.2. Paraffin-embedded sections and staining

1. 4% paraformaldehyde in 0.1 M phosphate-buffer (pH 7.4) (see **Note 4**).
2. Paraffin-embedded tissue blocks with fixed tissues.
3. LMD membrane slides covered with PEN-membrane (2.0 μm; Leica, No. 11505158), coated with poly-L-lysine (see **Note 5**).
4. Deparaffinization solution (xylene) and rehydration solutions (100% ethanol, 95% ethanol, 70% ethanol, and ultrapure water) (see **Note 6**).
5. Mayer's hematoxylin solution for staining paraffin-embedded sections.

2.3. Laser microdissection

1. Laser microdissection microscope system with UV-laser.
2. Collection tubes: autoclaved 0.2 ml PCR tubes with a flat cap (see **Note 7**).

2.4. RNA/DNA extraction kits

1. RNeasy Plus Micro Kit (Qiagen) for total RNA extraction.
2. miRNeasy Micro kit (Qiagen) for miRNA extraction.
3. High Pure PCR Template Preparation Kit (Roche) for genomic DNA extraction.
4. Qubit RNA HS Assay Kit (Invitrogen) for the measurement of RNA.

3. Methods

To achieve better results in the expression analysis using LMD samples, frozen sections of fresh tissues should be used (rather than formalin-fixed paraffin-embedded sections). This will preserve the nucleic acids from degradation. We use flash-frozen tissues to extract RNA and miRNA from LMD samples. For

genomic DNA analyses (cell-type specific genotyping), formalin-fixed paraffin-embedded sections are routinely used. The microdissection methods differ according to the microscope system that is used. Please follow the manual provided by the manufacturer for the precise microdissection method. We describe the method for LMD for the Leica LMD 7000.

3.1. The preparation of frozen sections for RNA or miRNA extraction

1. The tumor tissues of mouse models are dissected immediately after euthanasia. Wash the tissues briefly in PBS in a petri dish, immerse the tissue specimen in O.C.T. compound, and place it into an aluminum foil cup with O.C.T. compound (**Fig. 2**, see **Note 8)**.
2. Freeze the O.C.T. compound-embedded tissues in liquid nitrogen (LN_2) and store the sections in a deep freezer at -80°C (**Fig. 2**, see **Note 9**).
3. Adjust the temperature of the O.C.T. compound-embedded tissue block to -20°C. Cut the frozen block into 10-μm-thick slices using a cryostat, and immediately place each section on the RNase-free membrane of an LMD membrane slide (see **Note 10**). Let the frozen sections thaw to adhere to the membrane. If the cut sections are not used immediately, they can be stored at -80°C.

3.2. The fixation and staining of frozen sections

1. Fix the frozen sections on the membrane slides in an ice-cold ethanol and acetic acid mixture (19:1) solution for 3 min on ice by soaking in a 50-ml tube. Then, wash the sections in ultrapure water for 1 min on ice.

2. Proceed to stain the fixed sections in 0.05% Toluidine blue (pH 7.0) (see **Note 11**), and wash twice in ultrapure water for 1 min on ice.
3. Dry the fixed and stained sections using a slide glass dryer or hair dryer until the tissues are completely dried (**Fig. 2**). It usually takes less than 5 min. If the dried sections are not used immediately, they can be stored at -80°C.

3.3. Microdissection from frozen sections

We herein describe the method for LMD using a Leica LMD 7000.

1. Add 20 μl of lysis buffer from an RNeasy Micro Kit (Qiagen) or miRNeasy Micro Kit (Qiagen) mixed with 1% volume of β-mercaptoethanol (β-ME) (mix 10 μl of β-ME in 1 ml of extraction buffer) inside the caps of autoclaved PCR tubes, and place them in an appropriate location for laser microdissection (**Fig. 3**). The buffer is selected according to the purpose of the experiment.
2. Determine all of the cell compartments of interest, *i.e.,* stromal cells, cancer cells, or adjacent normal cells. Draw a line around the cell clusters of interest on the monitor screen, and cut the cell cluster out using a UV-laser. The dissected cells are then collected by gravity in the extraction buffer in the PCR tube cap (**Fig. 3**, see **Note 12**). Take photographs of the tissue sections before and after microdissection to confirm the cell-types that were collected (**Fig. 4**). If a fluorescent LMD system is used, then laser microdissection should be performed after fluorescence immunohistochemistry, which allows for the collection of more specific cell-types, including (but not limited to) Ki-67-positive proliferating cells, and F4/80-expressing TAMs.

3.4. The extraction of mRNA from frozen LMD samples

The extraction of mRNA is performed using an RNeasy Plus Micro Kit (Qiagen). All steps are performed at room temperature. The indicated buffers and columns are included in the kit.

1. Transfer solution including dissected cells from the PCR tube cap to a 1.5-ml tube containing 75 μl of lysis buffer (RLT), and mix well using a vortex for 30 s.
2. Transfer the lysate to a genomic DNA eliminator spin column (gDNA Eliminator column) to remove genomic DNA, and centrifuge at 8,000×g for 30 s. Collect the flow-through containing total RNA.
3. Add 75 μl of 70% ethanol to RNA solution, mix well by pipetting. Apply the cell lysate to the column (MinElute spin column), and centrifugate at 8,000×g for 15 s. Discard the flow-through.
4. Wash the column with 350 μl of washing buffer (RW1) by centrifugation at 8,000×g for 15 s, and discard the flow-through. Then, wash the column with 350 μl of washing buffer (RPE) by centrifugation at 8,000×g for 15 s, and discard the flow-through.
5. Wash the column with 500 μl of 80% ethanol by centrifugation at 8,000×g for 15 s, and discard the flow-through. After that, centrifuge again at 12,000×g for 2 min, and discard the flow-through.
6. Add 10 μl of autoclaved ultrapure water and elute total RNA by centrifugation at 12,000×g for 1 min.
7. Measure the RNA concentration using Qubit RNA HS Assay Kits and a Qubit 3.0 Fluorometer. The amount of RNA that can be expected to be extracted from LMD

sections is indicated in the **Table**.

3.5. The extraction of miRNA from frozen LMD samples

The purification of miRNA is performed using an miRNeasy Micro Kit (Qiagen). Perform all steps at room temperature. The indicated buffers and columns are included in the kit.

1. Add 200 μl of QIAzol Lysis Reagent in a 0.2-ml PCR tube containing dissected cells and mix well using vortex for 30 s.
2. Transfer the homogenized cell solution in the 1.5 ml tube and add 500 μl of QIAzol Lysis Reagent and mix well by vortexing for 30 s.
3. Add 140 μl chloroform to the tube and shake the tube vigorously for 15 s and centrifuge at 12,000×g for 15 min at 4°C.
4. Transfer the upper aqueous phase to a new 1.5 ml tube and add 525 μl (1.5 volume) of 100% ethanol and mix vigorously for 15 s.
5. Apply the sample to a column (RNeasy MinElute spin column) in a 2 ml tube and centrifuge at 8,000×g for 15 s at room temperature.
6. Add 700 μl of Buffer RWT to the column and centrifuge at 8,000×g for 15 s at room temperature.
7. Add 500 μl of Buffer RPE to the column and centrifuge at 8,000×g for 15 s at room temperature.
8. Wash the column with 500 μl of 80 % ethanol by centrifuge for 2 min at 8,000×g and discard the flow-through and centrifuge at 12,000×g for 5 min.
9. Add 10 μl of RNase-free water and elute the total RNA including the miRNA by

centrifugation at 12,000×g for 2 min and measure the RNA concentration. The amount of RNA that can be expected to be extracted from LMD sections is indicated in the **Table**.

3.6. Paraffin-embedded sections for genomic DNA extraction

1. Using 4% paraformaldehyde-fixed paraffin-embedded blocks, cut paraffin into 4-μm thick sections using a microtome, and put the section on the poly-L-lysin-coated LMD membrane slide.
2. Dry the sections overnight on a hotplate set to 37°C.
3. Deparaffinize the paraffin sections by soaking in xylene (5 min × 3 times), and then rehydrate the sections by soaking in 100% ethanol (5 min), 95% ethanol (5 min), 70% ethanol (5 min), and water (5 min).
4. Stain the sections with Mayer's hematoxylin solution for 5 min and wash the sections with water.
5. Dry the stained sections overnight on a hotplate set to 37°C (see **Note 12**).

3.7. Microdissection and the extraction of genomic DNA

This is a protocol for the isolation of genomic DNA from paraffin-embedded sections for genotyping by a PCR.

1. Add 20 μl of DNA extraction buffer from a High Pure PCR template purification kit to the cap of an autoclaved PCR tube, and place the tube in an appropriate location for laser microdissection (**Fig. 3**).

2. Determine the cell compartments of interest in the stained section, and draw lines around the cell clusters on the monitor screen, then cut the cell clusters out using a UV-laser. The dissected cells are then collected by gravity in extraction buffer in a PCR tube cap. Take photographs of the tissue sections before and after microdissection to confirm the cell-types that were collected.
3. Incubate the sections overnight with proteinkinase K at 5 mg/ml at 55°C to remove of proteins, including nuclease contamination; then inactivate proteinkinase K by incubation at 99°C for 10 min.
4. Add RNase at 1% vol and incubate at 37°C for 10 min to remove RNA, and then inactivate RNase by incubation at 70°C for 5 min.
5. Extract DNA according to the protocol provided by manufacturer. Usually, LMD samples from 3-5 sections are sufficient for the extraction of genomic DNA for genomic PCR by amplification of 200-300 bases.

4. Notes

1. O.C.T. compound embedding cups can be made from aluminum foil using various small bottles as templates (**Fig. 1**). After making the O.C.T. compound embedding cups, label the sample on the bottom of the aluminum cup for the purpose of identification. Without labeling, it is difficult to identify the tissues that are embedded inside the compound.
2. It is recommended that the LMD membrane slide be wiped with RNase AWAY (Molecular BioProducts) or any other RNase-removing solution to protect the frozen sections from RNase. RNase-free LMD membrane slides are also commercially

available.

3. The fixation solution is made by adding 38 ml of ethanol and 2 ml of acetic acid to a 50-ml conical plastic tube and mixing well. Two slide glasses (back-to-back) can be directly placed in this tube for fixation.
4. To make 0.1 M sodium phosphate buffer (pH 7.4), add 3.1 g of $NaH_2PO_4 \cdot H_2O$ and 10.9 g of Na_2HPO_4 to 1 L of distilled water. The pH should be adjusted to 7.4. Pour 100 ml of 0.1 M sodium phosphate buffer into a flask containing 4 g of paraformaldehayde. Cover with parafilm and put the flask in a 70°C water bath and mix until completely dissolved. This fixation solution should be freshly made.
5. Place a drop of Poly-L-lysin on the membrane of the LMD membrane slide and coat the surface with Kim wipes.
6. Pour xylene, 100% ethanol, 95% ethanol, 70% ethanol and ultrapure water into independent staining glass jars with lids. Prepare three sets of xylene.
7. These PCR tubes are required for the collection of microdissected tissues when using a Leica LMD 7000 (**Fig. 3**). Please follow the manual provided by the manufacturer for other LMD systems.
8. When you plan to make frozen tissue sections of stomach and intestine, the inside of the tissues should be washed with PBS using a 10-ml syringe with a needle, and then filled with O.C.T. compound using a 10-ml syringe without a needle. This process is particularly important for tissues from the gastrointestinal tract, as it avoids the development of bubbles in the embedded frozen blocks, which is a common issue in sectioning.

9. O.C.T. compound can be cracked by liquid nitrogen if the O.C.T. compound-containing aluminum foil cup is rapidly frozen in LN_2. To avoid cracking, prevent direct contact between the liquid nitrogen and the O.C.T. compound, O.C.T compound should be gradually frozen from outside for the first 2 min; then, the whole cup should be placed in LN_2 to ensure that it freezes completely (**Fig. 2A**). Alternatively, it is acceptable to only soak the bottom of the aluminum cup in LN_2 for the first 30 s, and then freeze the whole cup (**Fig. 2B**). Store the embedded frozen blocks at -80°C after labeling the bottom of the aluminum cups with indelible marker pen.
10. It is recommended that normal slide glass be used (not LMD membrane slide) for the first section to check the tissue morphology and to confirm that the required cellular compartments are contained within the section by Toluidine blue staining. After confirmation, cut the section, place it on a slide, and thaw it to adhere. We usually place three to five serial frozen sections on the same LMD membrane slide, depending on the sample size. In the case of a real-time RT-PCR, two slides (namely, up to 6-10 sections) are sufficient to analyze the expression levels of several independent genes. In contrast, more than five slides are required to extract a sufficient amount of RNA for a microarray.
11. It is crucial to check the Toluidine blue staining time because it can be difficult to observe the cell morphology in weakly or strongly stained sections. In the case of mouse stomach specimens, the slide should be loaded in Toluidine blue for 10 min to distinguish normal epithelial cells, tumor epithelial cells and stromal cells.
12. Paraffin sections should be dried completely before LMD because contamination with

residual water will affect the amount of recoverable DNA.

Table. The size of frozen section that are required for each of the experiments

Area (μm^2)	Concentration (ng/μl)	RNA amount (ng)	Purpose
200,000	0.05-0.2	0.5-2	miRNA qPCR
1,000,000	1-3	10-30	mRNAs qPCR
40,000,000	40-60	400-600	Microarray and RNA sequencing
70,000,000	180-210	1800-2100	

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Figure Legends

Figure 1. The preparation of aluminum foil cups for embedding frozen tissue in O.C.T. compound. (A) Prepare square-cut aluminum foil and various sizes of mini glass bottles. (B, C) Make aluminum foil cups using the bottom of the glass bottles, as indicated. (D) Label the bottom of the aluminum cups to identify the tissues.

Figure 2. The preparation of O.C.T. compound-embedded frozen sections. After cutting and wash with PBS, the tissue is placed in O.C.T. compound in an aluminum foil cup and then gradually frozen in LN_2, as described in the Methods (A), without directly touching the LN_2 or (B) by soaking initially only the bottom of the aluminum cup. (C) O.C.T. compound-embedded tissues should be stored in plastic bags at -80°C. (D) Dry the frozen sections using slide glass dryer.

Figure 3. An image of LMD sample preparation. When the Leica LMD 7000 is used, dissected tissue samples are collected inside the cap of a 0.2-ml PCR tube by gravity. Thus, lysis buffer or extraction buffer should be added to the inside of the cap before LMD.

Figure 4. Photographs of frozen sections before LMD (left) and after LMD (right). These photographs are particularly important to confirm and record the cellular compartments by LMD.

Figure 1

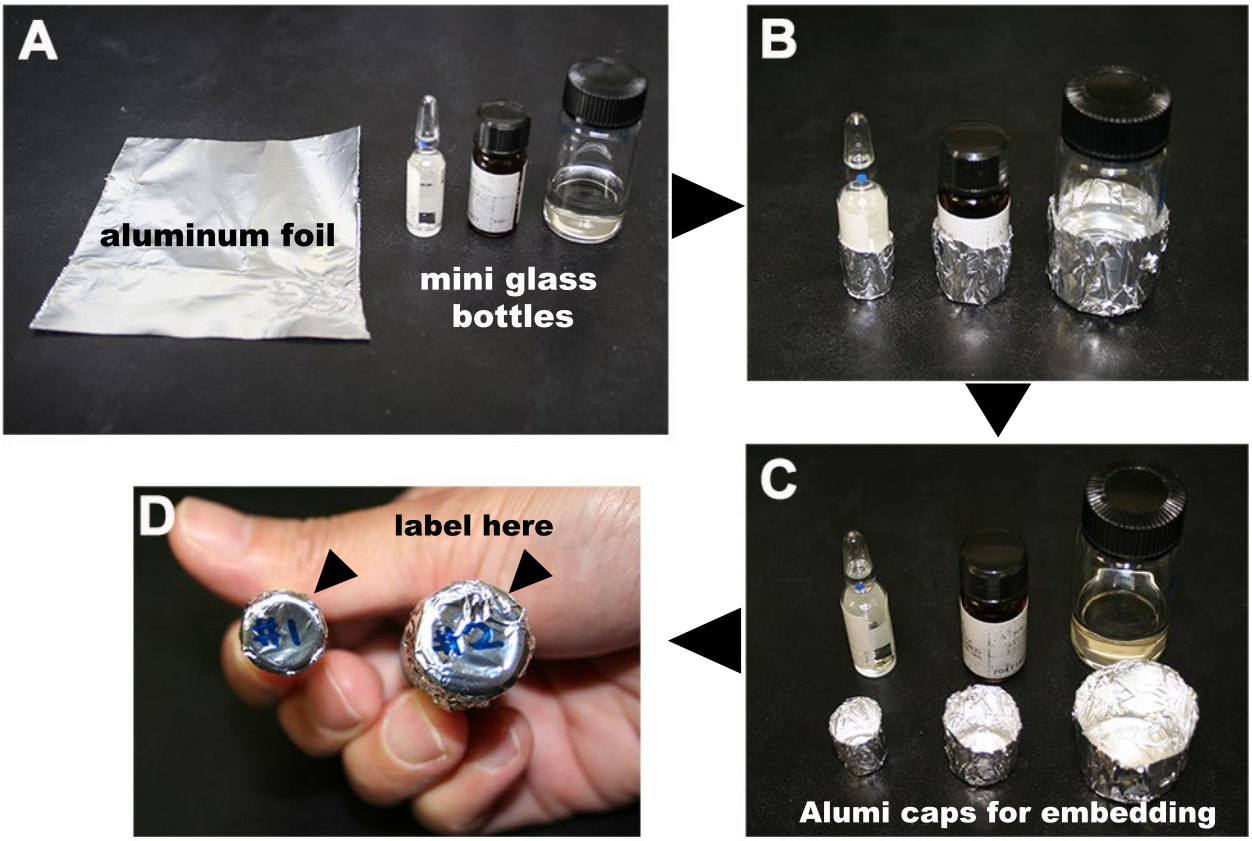
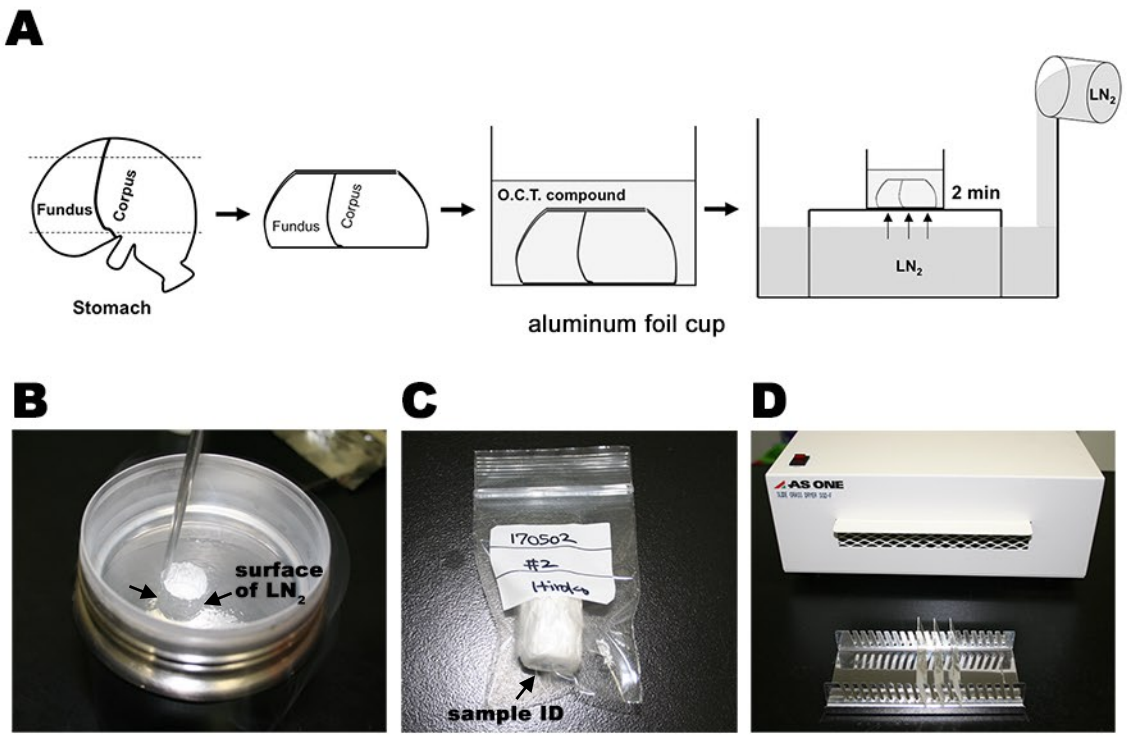

A
aluminum foil
mini glass bottles
B
C
Alumi caps for embedding
D
label here
#1
#2

Figure 2


A
Fundus
Corpus
Stomach
Fundus
Corpus
O.C.T. compound
aluminum foil cup
LN2
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B
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C
170502
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D
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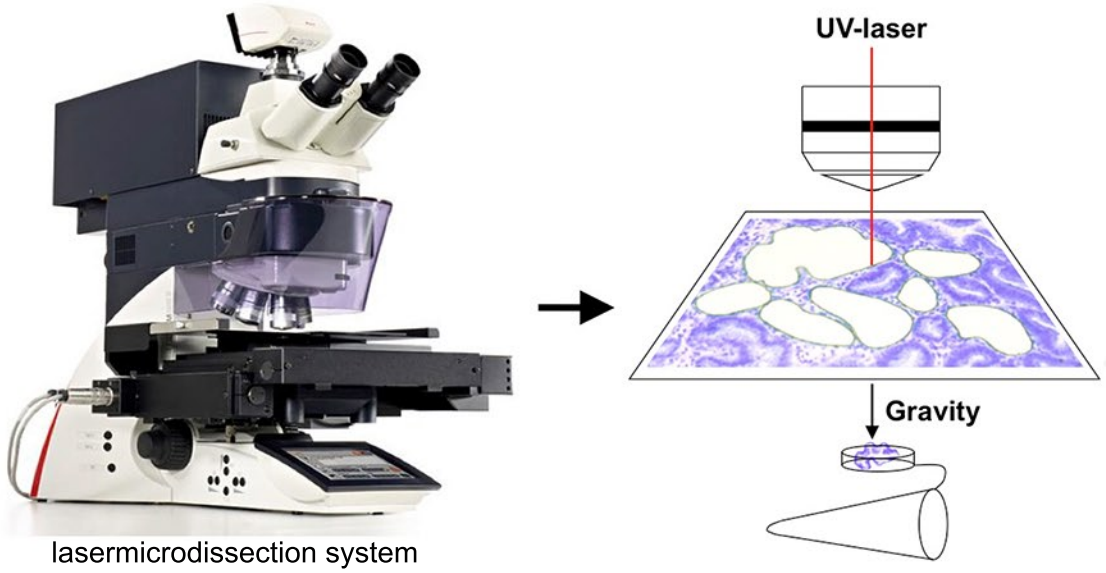
Figure 3



lasermicrodissection system

Figure 4