LAXCO
MICROSCOPES REDEFINED

Optimized Isolation of Live Plant Tissue using Laser Microdissection for use in Gene Expression Analysis

APPLICATION NOTE #9

ABSTRACT

Genetic expression in colonic epithelial cells, responding to the changes of human intestinal flora altered by exposure to drugs or contaminants, provides valuable health information. Gut flora are important in: (1) Metabolic fermentation and energy salvage, (2) Intestinal cell proliferation and differentiation, and (3) Development and homoeostasis of immune system and protection against pathogen invasion. Many drugs orally delivered or present in food may disturb normal flora and cause human health concerns.
Here we report a short, but integral method to understand host-microbial relationships in the intestine, as well as to identify the genetic basis for the impact of drugs on intestinal functions. This is achieved by comparing gene expression in colonic epi- thelial cells between germ-free (GF) mice, human flora-associated (HFA) mice and drug-treated HFA mice using laser capture microdissection, RiboAmp® HS RNA Amplification Kit and cDNA microarrays.

INTRODUCTION

Live plant tissue is thick (300-500 µm thick) and contains moisture, characteristics which typically inhibit effective laser capture microdissection. The UV laser of the VeritasTM Microdissection System has provided a means to cut through the difficult live plant tissue. Optimizations of the technique, detailed herein, have been made to enhance the process.

For microdissection, live plant tissue is mounted on the underside of a polyethylene-naphthalate (PEN) frame membrane slide, and the LCM cap adheres to the membrane and the tissue for capture.

Presumably due to the mass of the cut tissue, large regions have proven difficult to keep in contact with the LCM cap. To ensure pick up of the thick, wet tissue, a number of different slide adhesives were applied to the membrane of a frame slide to improve capture of the live tissue, including nail polish, which has traditionally been used in microscopy to seal coverslips onto slides. Here we show that both nail polish and a common glue stick are effective adhesives for live tissue laser capture. Further, we demonstrate success in RNA isolation from live plant tissue using the Veritas Microdissection System for use in reverse transcription PCR (RT-PCR).

MATERIAL

- PEN Membrane Frame Slide (ThermoFisher Catalog #LCM0521)
- Plain glass slides
- Quick drying clear nail polish
- Glue stick
- Poly-lysine (50 µg/mL) (Sigma Aldrich Catalog # P6407)
- Chrome alum gelatin (chromium potassium sulfate dodecahydrate) (Sigma Aldrich Catalog # 24336-1)
- CapSure® Macro LCM caps (ThermoFisher Catalog #LCM0201)
- VeritasTM Microdissection System (ThermoFisher Catalog #LCM1704)
- PicoPure® RNA Isolation kit (ThermoFisher Catalog #KIT0204)
- Trizol® (Invitrogen Catalog #15596-026)
- Quant-iTTM RiboGreen® RNA Assay Kit (Molecular Probes Catalog #R11490)
- Wallac 1420 Microtiter Plate Reader (Per- kin Elmer Wallac, Turku, Finland)
- Black Microtiter plates (VWR International 62402-983)
- GeneAmp® 0.5 mL tubes (Applied Biosys- tems Catalog #N8010737)
- SensiscriptTM kit (Qiagen Catalog #205211)
- REDTaqTM ReadyMixTM PCR Reaction Mix (Sigma Catalog #R2423)
- Specific PCR primers (Keck oligo synthe- sis, Yale University)
- 1 kb DNA Ladder (NEB N3232L)
- BioRad Gel Doc 2000 Documentation System (BioRad, Hercules, CA)
- Live plant material

METHOD

Plant Material Preparation:

Sections of leaves from Arabidopsis thaliana, Nicotiana tobaccum (tobacco) and Oryza sativa (rice) were manually cut with a razor blade.

Slide Preparation:

1. Prior to placing the plant tissue on the PEN membrane frame slides, each slide was coated with various adhesives (water, polylysine, gelatin, glue stick, quick drying clear nail polish) to determine the optimum treatment for improved microdissection.

   Note: If desired, the slides may be treated for 30 minutes with UV irradiation prior to treating with additional adhesives. The UV exposure helps to sterilize the slide as well as to improve the adherence characteristics.

2. Water, glue and nail polish were each spread lightly directly onto the membrane of separate clean frame membrane slides, immediately before applying the tissue.
3. Alternately, a clean frame membrane slide was coated with 50 µg/ml polylysine and allowed to incubate at room temperature for 30 minutes, after which the solution was removed and the slide air dried before use.
4. Gelatin slides were prepared by dipping the slides three times in chrome alum gelatin followed by drying overnight before use.
5. The plant tissue was placed on the flat surface of a treated PEN Membrane Frame Slide and then a plain glass slide was placed on top of the plant tissue, forming a “frame slide sandwich”. The “sandwich” then placed in the Veritas Microdissection System glass slide down (Figure 1).

   Note: The plant tissue must be placed on the flat side of the slide membrane to facilitate microdissection (not the “well” side).

Laser Cutting and Laser Capture Microdissection:

Following the protocol detailed below, microdissection was performed for each tissue preparation to determine the ideal adhesive for consistent success.

1. The laser capture (IR laser) properties were set to the following: 70 mW power, 4500 msec duration, 2 hits with 2 msec delay.
2. The UV laser power was adjusted according to the tissue thickness. The UV laser was set at 65 for Nicotiana tobaccum leaf, which was the thickest tissue. Arabidopsis and Oryza leaves, which are thinner than tobacco, could be cut with the laser set at 20-45. Thicker tissue required higher UV laser settings to achieve complete cutting through the tissue.
3. CapSure® Macro LCM caps were used for laser cut and capture of all plant tissues.
4. UV cutting was repeated at least once to ensure complete cutting, and the cut site inspected to see if further spot cutting was necessary.
5. Disks of 50 -1600 µm diameter were cut and captured onto separate caps (Figure 2).

Note: It is important that the tissue be cut on all sides to ensure successful detachment from the neighboring cells.

RNA Isolation and Quantitation:

Once the optimal adherence medium was determined (glue stick and nail polish), the associated microdissected disks were extracted using two different extraction methods: Trizol and a GITC-based isolation using the PicoPure® RNA Isolation Kit.

1. Extraction methods were evaluated on isolated Arabidopsis and Tobacco leaf disks, each with a diameter of about 1600µm.
2. 100 µL of appropriate extraction buffer (PicoPure XB or Trizol) was pipetted into a GeneAmp 0.5 mL tube.
3. Each CapSure Macro LCM cap, containing one microdissected plant tissue disk, was placed into a tube con taining extraction buffer.
4. The tubes were inverted to ensure the cap surface was completely covered by the extraction buffer.
5. Each cap-tube assembly was vortexed and then frozen at –80 °C. Tubes were frozen for a minimum of 15 minutes and up to one week.

Note: This freeze / thaw step helps to break down the cells, allowing the buffer to penetrate more easily.

6. PicoPure extracts:

6.1 Samples were thawed.

6.2 The tubes were incubated at 42 °C for 30 minutes.

6.3 100 µL of 70% ethanol was added to each tube, then mixed with a pi- pette.

6.4 All 200 µL of the mixture was added to the PicoPure purification column.

6.5 The remainder of the procedure followed the recommended manufacturer’s protocol.

7. Trizol extracts:

7.1 7.2

Samples were thawed and vortexed.

An additional 200 µL of Trizol was added to each tube.

Figure 1. Laser cutting using the frame slide "sandwich".

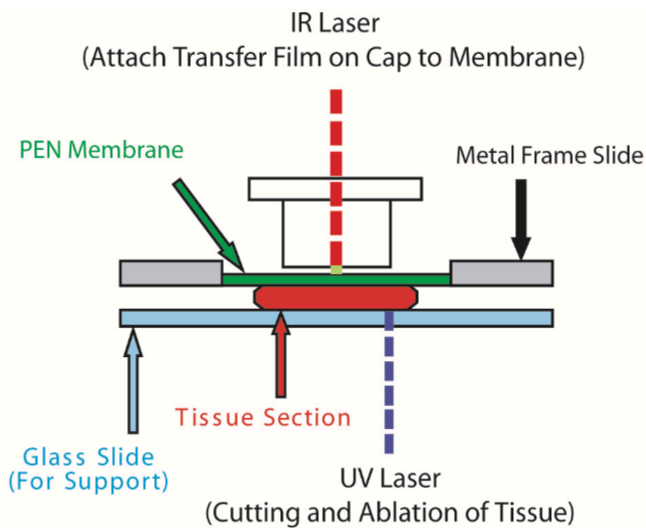
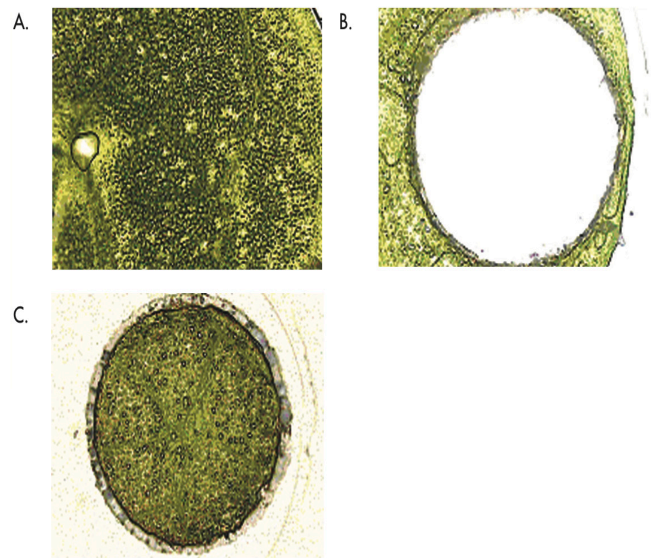


Figure 2: Live capture from Arabidopsis thaliana leaf. A. Before microdissection; B. After microdissection; C. Captured live tissue on CapSure LCM cap.



7.3 The tubes were vortexed and then incubated at room temperature for five minutes in an upright position.

7.4 60 µL of chloroform was added to each tube. Tubes were shaken vigorously for 15 seconds, then incubated at room temperature for three minutes.

7.5 The tubes were centrifuged at 12,000 x g for 15 minutes at 4°C.

7.6 The colorless upper phase was re- moved from each tube and trans- ferred to tubes containing 150 µL of isopropyl alcohol, then incubated at room temperature for 10 minutes.

7.7 The tubes were centrifuged at 12,000 x g for 30 minutes at 4°C to pellet the RNA.

7.8 The alcohol was drawn off and rinsed with 75% ethanol.

7.9 To re-pellet the RNA, the tubes were centrifuged at 7,500 x g for 5 minutes at 4°C.

7.10 The alcohol was removed and the pellets were allowed to air-dry brief- ly.

7.11 The pellets were resuspended in 10 µL RNase-free water.

8. Total RNA (1 µL) from each sample was quantitated using the RiboGreen assay (Invitrogen / Molecular Probes).

RT- PCR:

Arabidopsis, a well-studied plant model system, was used to assess the feasibility of RT-PCR using RNA isolated from the microdissected live plant sections. Extracts from tissue isolated using the nail polish coated PEN frame membrane slide were used for the following experiment.

1. Total RNA (10 ng) isolated from each Arabidopsis tissue disk was reverse transcribed using the Qiagen Sensiscript kit following the manufacturer's instructions.

2. Equal amounts of cDNA (5 µl) were used for PCR.

3. Two different primer sets (Actin 8 and Suc2) were used. Red Taq Mix (Sigma) was used in a total volume of 25 µl.

4. Thermocycler conditions were as follows: 94 °C 2min, 35 cycles of 92 °C 15 sec, 55°C30sec,72°C45 sec with a 10min extension at 72 °C.

5. PCR products were run on a 0.8% gel in 1X TAE along with 1 kb DNA ladder and visualized using ethidium bromide and a BioRad Gel Doc 2000.

RESULTS

Despite live plant tissue being thick and wet, we were able to successfully and cleanly microdissect tissue samples from all the live plant tissues that were sampled. Various adhesives were tested to determine which most successfully ensures adherence of the live plant tissue to the frame membrane for successful transfer during LCM cap transport. Of the different adhesives used, the nail polish and the glue stick performed the best: each successful in 9/10 trials. The other treatments worked approximately half the time and therefore were not selected for further use. The nail polish helped the thick tissue stick to the membrane without interference to the UV or IR lasers. The glue stick also kept the tissue attached to the membrane and cap; however the appearance of the glue was noticeable on the tissue when the cap was viewed under the microscope. It is possible that the presence of the glue or the nail polish on the isolated tissue disks may affect the isolated RNA, in a manner not detected by RT-PCR, which can function even with somewhat degraded RNA. The procedures used to label, amplify or otherwise manipulate the RNA other than for RT-PCR, may be affected by the presence of either nail polish or the glue. We are not sure if any of the adhesive agents are carried through the RNA isolation procedure. Further study is needed to determine potential impact of these adhesive agents on other procedures used for gene expression. In this experiment, there seems to be no inhibitory affects on RNA isolation or RT-PCR.

The captured tissue disks were processed for RNA isolation using either Trizol or the PicoPure RNA isolation kit from ThermoFisher. PicoPure RNA reproducibly yielded greater amounts of total RNA. Average yield for duplicate samples extracted using PicoPure was 28 ng / 1600 µm tobacco disk versus 18 ng / 1600 µm using the Trizol method.

Two genes were chosen to demonstrate the compatibility of microdissected live plant material with RT-PCR analysis; Actin 8 and Suc2. RNA from microdissected disks of Arabidopsis leaf (chosen as it is a well studied plant model system) with and without vein tissue, was analyzed for the expression of each gene. Actin 8, highly conserved and critical in cytoskeleton formation, is expressed in all cells and was run as a positive control. Suc2, encoding a sucrose H+ symporter, exhibits vein-specific expression and therefore was expected in only those samples containing vein tissue.

All samples showed bands for Actin 8 and as anticipated, only the sample lacking vein tissue did not demonstrate expression of the Suc2 gene (Figure 3).

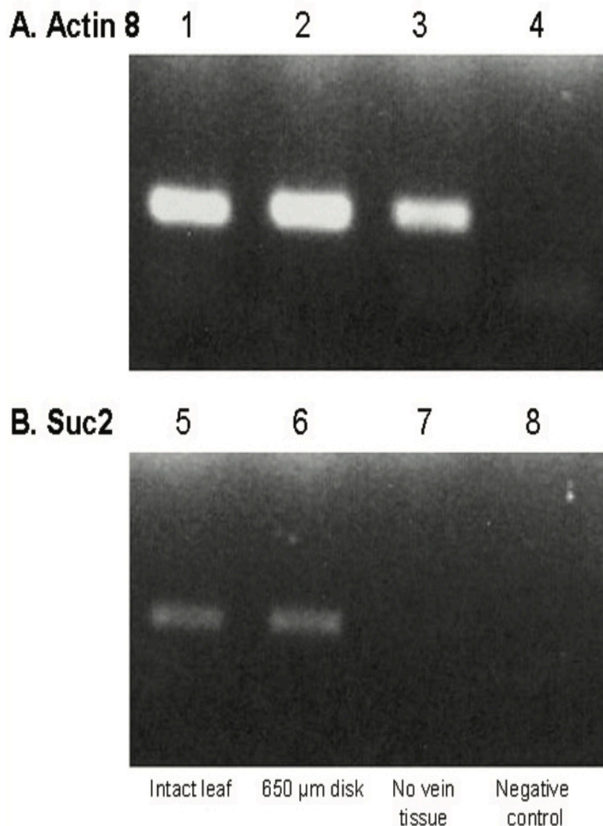
CONCLUSION

We have shown that it is possible to successfully isolate RNA from live plant tissue that has been microdissected using the UV laser of the Veritas Microdissection System. RNA isolated using the PicoPure RNA Isolation kit proved superior to a Trizol method and sufficient to perform gene expression studies using RT-PCR. Further, this study supports previous experiments that have shown no great loss or alteration of the RNA due to the laser. In an earlier evaluation of tissue microdissected with and without UV laser, comparable yields were obtained: 2.7 ng / 500 cells with UV laser vs. 3.0 ng / 500 cells without UV laser (Tausta unpublished data. Data not shown herein).

Figure 3: RT-PCR using RNA from live plant tissue.

Panel A is an agarose gel of RT-PCR products using Actin-8 specific primers and panel B is an agarose gel of RT-PCR products generated from the same RNA samples, but using Suc2-specific primers. Suc2, encoding a sucrose H+ symporter, exhibits vein-specific expression.

Lanes 1 and 5: Total RNA extracted from three intact Arabidopsis leaves. Lanes 2 and 6: Total RNA extracted from 3 Arabidopsis leaves from which 10 disks of about 650 um were microdissected. Lanes 3 and 7: Total RNA extracted from one 650um diameter Arabidopsis leaf devoid of vein tissue. Lanes 4 and 8: Negative control; no input RNA.



It should be noted that to obtain a pure cell population, it is still optimal to use plant tissue sections, as the use of live plant tissue provides only a heterogeneous population of cells multiple cell layers collected at once. In some cases, it may be possible to use specific live sections, such as epidermal peels, to eliminate undesired material, but this is still not the optimal manner by which to ensure a homogeneous cell population.

Combined UV laser cutting and LCM enables the isolation of live tissue exhibiting live markers such as GFP, metabolites and other chemicals that might otherwise be lost or destroyed during normal fixation. It also allows for the isolation and capture of physically identifiable structures that may otherwise be compromised during normal LCM procedures. Further studies are needed to optimize conditions, but it appears evident that microdissected live plant tissue can be processed for RNA, DNA and protein or metabolite isolation and subsequent downstream analysis.

REFERENCES

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