Video Article

A Novel Microdissection Approach to Recovering *Mycobacterium tuberculosis* Specific Transcripts from Formalin Fixed Paraffin Embedded Lung Granulomas

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Abstract

Microdissection has been used for the examination of tissues at DNA, RNA, and protein levels for over a decade. Laser capture microscopy (LCM) is the most common microdissection technique used today. In this technique, a laser is used to focally melt a thermoplastic membrane that overlies a dehydrated tissue section[1]. The tissue section composite is then lifted and separated from the membrane. Although this technique can be used successfully for tissue examination, it is time consuming and expensive. Furthermore, the successful completion of procedures using this technique requires the use of a laser, thus limiting its use. A new more affordable and practical microdissection approach called mesodissection is a possible solution to the pitfalls of LCM. This technique employs the MESO-1/MeSectr system to mill the desired tissue from a slide mounted tissue sample while concurrently dispensing and aspirating fluid to recover the desired tissue sample into a consumable mill bit. Before the dissection process begins, the user aligns the formalin fixed paraffin embedded (FFPE) slide with a hematoxylin and eosin stained (H&E) reference slide. Thereafter, the operator annotates the desired dissection area and proceeds to dissect the appropriate segment. The program generates an archived image of the dissection. The main advantage of mesodissection is the short duration needed to dissect a slide, taking an average of ten minutes from set up to sample generation in this experiment. Additionally, the system is significantly more cost effective and user friendly. A slight disadvantage is that it is not as precise as laser capture microscopy. In this article we demonstrate how mesodissection can be used to extract RNA from slides from FFPE granulomas caused by *Mycobacterium tuberculosis (Mtb)*.

Video Link

The video component of this article can be found at https://www.jove.com/video/51693/

Introduction

Samples have traditionally been manually microdissected from either whole tissue or slides using a needle and scalpel. This necessitates a clear separation between the tissue section of interest and surrounding tissues sections[2]. With the current advancements in molecular profiling technology, there has been an increasing need to assess tissue at the cellular level. Due to the limitations of manual microdissection, techniques including LCM were established to allow greater isolation precision. This technique allows the researcher to isolate specific cell populations from various cell and slide types, which can then be used for downstream assays such as gene expression profiling. Although very effective for downstream assays, LCM is not without limitations. First, LCM is an expensive and time consuming process. Additionally, due to the unstable nature of RNA, it is often challenging to obtain high quality RNA from LCM samples[2]. Due to the disadvantages of LCM, new advances in microdissection technology are still needed to make it more accessible to a greater number of researchers in an affordable and time sensitive manner.

One such advancement in microdissection technology now available is a technique known as mesodissection. In this technique a machine is used to mill the annotated tissue section of interest and aspirates it into a consumable mill bit[3]. Next, this sample can be aspirated into a collection tube and used for downstream applications. The advantages of this system are that it is significantly less expensive and time sensitive. In our experience, the system allows the dissection of a lung granuloma tissue section in ten minutes. On the other hand, traditional LCM would likely require hours to complete the process. The system allows the operator to load a reference slide to use as a comparison as well as tool for annotation. Additionally a report is generated outlining the area of the slide that was dissected. There are two main disadvantages to mesodissection. Although effective at extracting multiple cells, it is challenging to isolate a single cell. In addition, the precision of the image generated is not as clear as if using other imaging microscopes.

Tuberculosis (TB) is a major infectious disease killer of humanity worldwide and results from infection with *Mtb*. In a majority of individuals exposed to aerosols of *Mtb*, the infection is latently limited. In at least 10 million people annually, it results in active TB disease[4]. During latent

infection, *Mtb* is contained within pathological lung lesions known as granulomas. Hence it has been argued that the outcome of *Mtb* infection is decided at the level of the granuloma[5].

Here we demonstrate how mesodissection can be used to microdissect granulomas caused by *Mtb*. The slides used are from FFPE lung tissue from infected rhesus macaques. For the purpose of this demonstration, we will dissect granulomas in their entirety. We also demonstrate that RNA can be extracted from the recovered tissue. This technique can be applied to tissue samples from various other specimens and then used for a variety of downstream assays.

Protocol

1. Calibrate Mesodissection Instrument with 2iD Imaging Software

The 2iD imaging software will be referred to as either the software or the program for the remainder of this article. This step is needed to correctly track the image as well as align multiple slide frames.

1. Turn on instrument and computer.
2. Open software and select “calibrate instrument”.
3. Calibrate stage travel by using the joystick. Move stage to upper left position. Press “set” under step 1 in program.
4. Move stage to lower right position. Press “set” under step 2 in program.
5. Calibrate aspiration by first pressing “Z-button” to raise head assembly. Press “aspirate pulse” button and “dissection” button on the joystick until plunger control rod reaches top position. Press “set” under step 3 of program.
6. Press “aspirate reset” button. Once the plunger stops at the bottom position press “set” in step 4.
7. Click on the “scale tab”.
8. Place calibration ruler on blank side.
9. Focus microscope if needed.
10. Draw line between the two scale bars using mouse.
11. Enter distance in box labeled step 2. Press “compute” button.
12. Click on “crosshair” tab. Turn motor speed to 1.
13. Insert xScisor into head assembly. Lower head assembly into z-axis ready position by pressing “z-axis” button. NOTE: The head assembly is the large structure on the top the dissection instrument. The xScisor will be referred to as a consumable mill bit for the remainder of this article.
14. Click “dissection engage” button on joystick. NOTE: This button is located on the top of the joystick.
15. Define center of rotation visually. To do this, move center of crosshair over center of rotation using defined parameter in step 1 of program. Use the up and down arrows in the “x” and “y-axis” bars to adjust the pixels to align crosshair. Click the “done” tab.
16. Click on “home” icon. NOTE: This is represented by a house image located in the upper right hand corner.

2. Create Reference Image

1. Place H&E slide on stage. Place opaque cover on top of slide. Drive the instrument by moving the joystick to the desired area of the H&E slide. Save the image generated. NOTE: Either the “capture reference” tab or “dissect” tab can be used to complete this step.

3. Align Tissue for Dissection

1. Click the “dissect tissue” option on the home screen.
2. Fill out the “operator,” “dissection accession number,” “reference accession number,” “xScisor barcode number,” “dissection fluid lot number,” “xScisor size” and “description” in the “setup” tab.
3. Click on the “find tissue” tab.
4. Import previously saved reference image.
5. Place an unstained FFPE slide of 5 micron thickness on the stage. Move stage to the same area as the one depicted in the reference image. Click on “insert image” tab.
6. Click on “align” tab. Use the mouse and associated arrows to align the reference image to the unstained image.

4. Annotate Slide

1. Click on the “annotate” tab.
2. Use the mouse to draw a circle around the desired area of the slide that will be microdissected.

5. Load Consumable Mill Bit

1. Fill consumable mill bit with desired buffer, here PKD buffer, by pulling the plunger up and down in a fluid motion several times. Make sure to exclude air bubbles.
2. Raise Z-axis by pressing “Z-axis button”.
3. Load consumable mill bit into machine. Do this by sliding the consumable mill bit into the top of the machine so that the white line on the instrument lines up with the black line on the consumable mill bit. Press “aspiration reset” button to allow plunger to be lowered into the correct position. NOTE: It should be easy to slide the consumable mill bit. If it is not, make sure the notches are correctly aligned.

4. Lower Z-axis by again pressing “Z-axis” button.

6. Dissect Tissue (Figure 4)

1. Open “dissect” tab.
2. Turn motor and aspiration speeds to 1.
3. Check “show tracking” box. Note: This allows the user to visualize the dissected area during the dissection process.
4. Once ready to dissect, continue to hold “engage” button as well as “aspirate” button while moving joystick in a counterclockwise direction to dissect tissue.
5. Continue to dissect in a counterclockwise direction until the aspirate is “full” tab turns red.

7. Empty and Remove Consumable Mill Bit

1. Place a 0.5 µl microfuge tube under the tip of the consumable mill bit.
2. Press “aspiration” control rod quickly to eject sample into microfuge tube.
3. Press with increased force to eject used consumable mill bit.
4. Rinse tissue fragment by pulling plunger back. Push plunger forward to express remaining sample into consumable mill bit. The tissue fragment will now be contained within the microfuge tube.

8. Lyse Sample with Proteinase K Digestion Followed by Heat Treatment

1. Add 70 µl TE pH 8.5 containing 5 µg of proteinase K to recovered tissue sample.
2. Place sample into programmable heater-shaker.
3. Complete digestion using programmable heater-shaker at the following setting: 60 ˚C for 30 min at 1,500 rpm; 82 ˚C for 15 min at 1,500 rpm; and 25 ˚C for 1 min at 450 rpm. The sample is now ready for downstream applications.

Representative Results

The aforementioned protocol shows how to use a new mesodissection technique to extract RNA from FFPE tissue slides. This protocol’s efficacy is shown through FFPE slides of lung granulomas from NHP infected with *Mtb* in various infective stages. **Figures 1-3** are images of the instrument. **Figure 4** depicts how the dissection process occurs and the resulting image generated by the software. **Table 1** shows the results of RNA extraction with a granuloma from a NHP at each infective state. RNA was amplified and purified to allow for RT-PCR evidence of 16s (**Tables 2-4**). **Table 4** confirms the presence of *Mtb* ribosomal subunit 16s, and thus *Mtb*, in the microdissected samples.

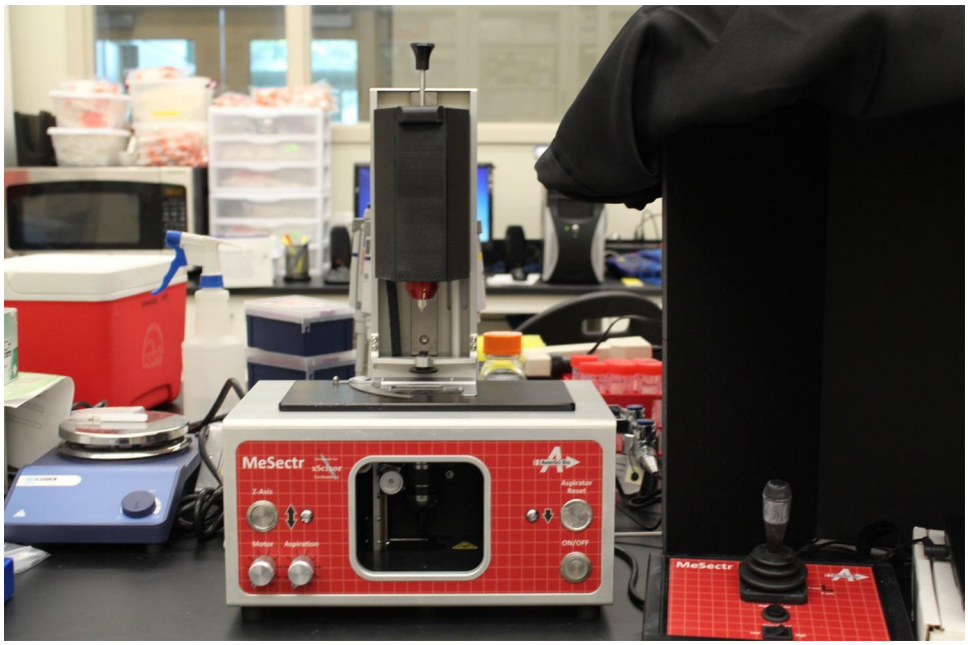

Figure 1. Image of mesodissection instrument including joystick. Please click here to view a larger version of this figure.

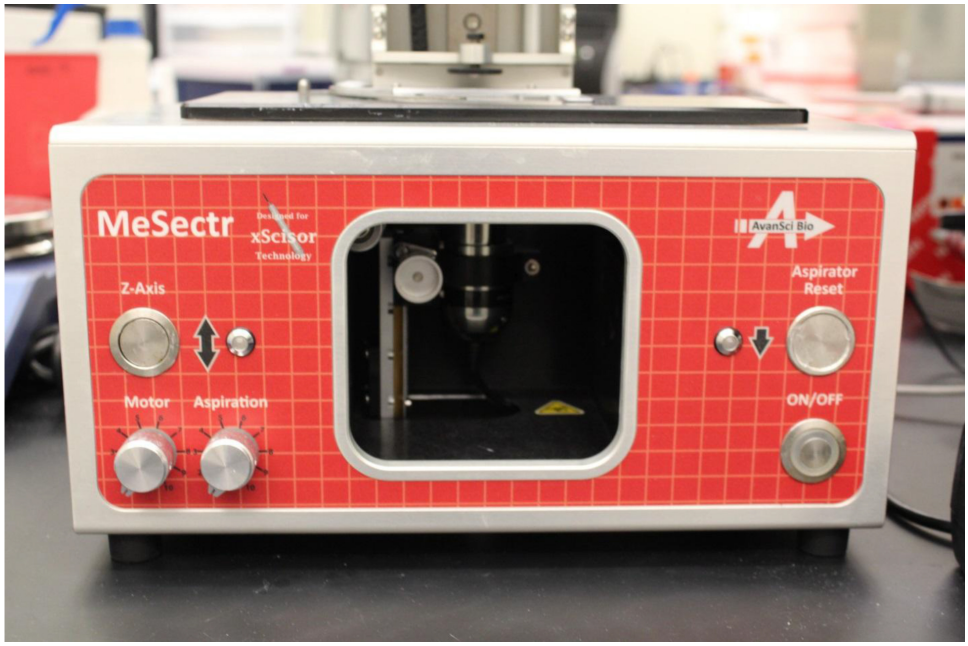

Figure 2. Closer view of mesodissection instrument. The “z-axis” button, “motor” speed and “aspiration” speed knobs are shown on the left side of the image. The “aspirator reset button” and “on/off” button are shown on the right side of the image. The slide stage is on the top of the instrument. Please click here to view a larger version of this figure.

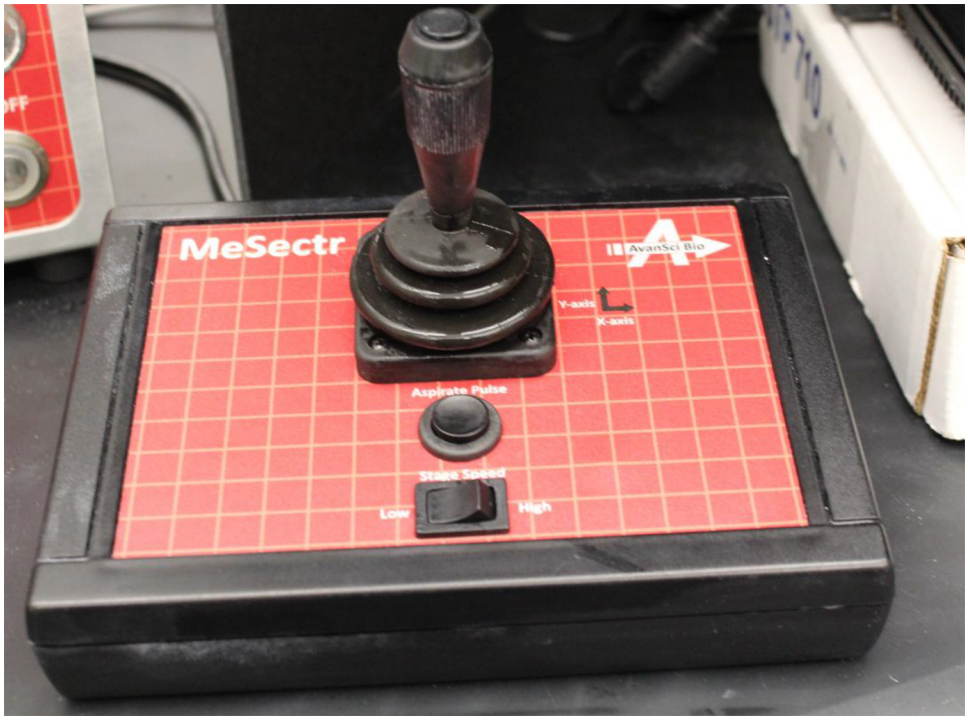

Figure 3. Closer view of joystick. The joystick, “aspirate pulse” and “stage speed” buttons shown. Please click here to view a larger version of this figure.

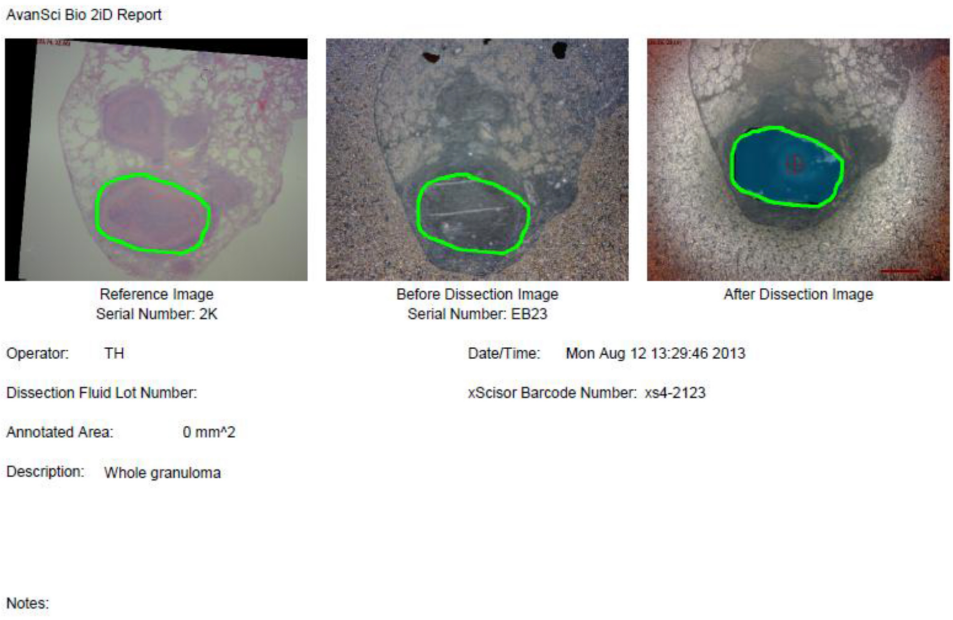

Figure 4. Report of whole granuloma mesodissected from NHP EB23. Animals used in this study were infected with CDC1551 *MTb* via aerosol. The reference image on the left shows an H&E stained granuloma from an actively infected rhesus macaque. The desired area of interest to be dissected is outlined in green. The image in the middle shows the corresponding unstained FFPE slide of 5 micron thickness before dissection. The areas were aligned using the software and the correlating area of interest is outlined in green. The image on the right depicts the same unstained FFPE slide post dissection. The area dissected is represented in blue. A 400 mm^2 consumable mill bit was used for the dissection, which is designed for traverse dissection of medium to large dissection area. Large dissection area is described as less than 100 mm^2. Sizes designed for other areas/dissection types are available. Although the report describes the annotated area as 0 mm^2, this is inaccurate. When we remove the microdissected slide from the machine and physically look at the area of interest itself, we can see the corresponding area on the slide that no longer has tissue and therefore has been dissected. It should be noted that the authors are renting the instrument and this issue has been fixed on other instruments. Please click here to view a larger version of this figure.

Primate	Infective State	RNA concentration in ng/µl	Total RNA in ng	260/280	260/230
EB23	Active	48.3	724.5	1.71	1.59
HB12	Latent	61.4	921	1.7	1.86
HP41	Reactivated via co infection with SIV	22.3	334.5	1.9	2.18

Table 1. RNA concentrations post extraction. The sample was DNAsed and RNA extraction was performed using the Qiagen RNAeasy FFPE kit. RNA concentration was obtained using a nanodrop. NHP infective stages are also depicted. RNA was eluted in 15 µl RNAse free water.

Primate	Infective State	cDNA concentration in ng/µl	Total cDNA in ng	260/280	260/230
EB23	Active	31.7	951	2.08	2.37
HB12	Latent	156.5	4695	1.94	4.4
HP41	Reactivated via co infection with SIV	41.7	1251	2.19	3.44

Table 2. cDNA concentrations post amplification and purification. NHP infective stages are also depicted. RNA was amplified using Ovation RNA-Seq FFPE System (Part no. 7150) and resulting amplified cDNA was purified using the QIAGEN QIAquick PCR Purification Kit as suggested by Nugen on page 26 of the amplification system's user guide. cDNA was eluted in 30 µl TE buffer.

Well	Reporter	Ct	Tm value
16S 10-1	Standard	12.35138	79.4
16S 10-2	Standard	16.144611	79.4
16S 10-3	Standard	17.911345	79.4
16S 10-4	Standard	21.762596	79.4
16S 10-5	Standard	25.746624	79.4

16S 10-6	Standard	28.505266	79.1
HB12	Unknown	25.771492	77.8
HP41	Unknown	17.760149	78
EB23	Unknown	24.754618	78.2
Negative Control	NTC	33.544422	79.7

Table 3. RT-PCR results with respect to 16s ribosomal subunit. Appropriate amplification observed thus proving the presence of *Mtb* within samples. Genomic DNA from CDC1551 strain *Mtb* was used as a standard.

Discussion

Mesodissection is a technique that can be applied to RNA extraction from pathologic lesion slides caused by a vast array of pathogens. It is a necessity that the user tracks the image and aligns the image correctly. To accomplish this, the instrument must be calibrated following the directions on the imaging software. When dissecting, if the slide and area of interest being dissected do not align, the user must recalibrate the instrument. Another critical step in the dissection process is to load the consumable mill bit in a manner such that it is devoid of bubbles prior to dissection (Step 5.1). We have found that the presence of bubbles decreases the dissection yield. To correct this problem, we recommend propelling and expressing the plunger multiple times. The user can practice this by adding food coloring to a practice buffer to visualize bubbles, as recommended by the manufacturer. Once effective, the user should then proceed with normal dissection. The last critical step is to dissect the sample in a counterclockwise direction. The machine mills in a counterclockwise direction; therefore, we have found that moving the joystick in a circular motion in a counterclockwise manner increases tissue yield as the leading edge cuts more cleanly than the trailing edge[6]. Another way to improve tissue yield is to decrease the speed of the dissection process. We have found that setting the aspiration rate and motor speed on the lowest speed while simultaneously setting the stage speed to “low” produce the highest tissue yield. There are two main limitations to this microdissection approach. The first limitation is that we have found point dissection very challenging due to the need to drive the joystick in counterclockwise circles in the dissection process. The second limitation is that the camera on the instrument generates an image that lacks the cellular detail of an image generated from an advanced microscope.

Mesodissection has direct implications with respect to transcriptomic analysis of *Mtb*. Granuloma formation is a hallmark of TB infection and results from the host’s attempt to contain the pathogen within a confined region of lung[5]. Conversely, it has been proposed that *Mtb* has evolved to allow its persistence in granulomatous lesions[7]. *Mtb* is subjected to different stresses depending on the stage of infection. Some of these stresses include but are not limited to redox stress, low pH, membrane damage, hypoxia, lack of nutrition, etc[7-13]. Throughout these various stages, *Mtb* can still survive in a latent form and even reactivate. This means that different genes are upregulated and downregulated at different infective stages. For example, *Mtb* encodes for over 200 transcription factors[4]. In addition, it has been shown that the expression balance between different chemokines, such as α and β chemokines, within the granuloma may be important protection markers[8]. Evaluation of mycobacterial transcriptomics in granulomatous tissue is likely to further our understanding of the mechanisms involved in their formation and maintenance as well as those genes that are expressed in each state of the infection. This will allow us to evaluate mycobacterial transcriptomics in various TB infective stages including active, latent and reactivated disease as well as various regions within the granuloma itself. In addition to allowing us to further assess mycobacterial transcriptomics, the above procedure also allows for the assessment of host transcriptomics in the same infective states. Furthermore, this approach then can be used to compare, contrast, and analyze the relationship between host and bacteria.

Although an important tool in TB research, the aforementioned protocol can be applied to other pathogens as well. The above protocol uses FFPE slides from infected lung samples, but can also be applied to lesions from other organs. We demonstrate how to use the system to extract RNA, but it can theoretically be used for DNA and protein extraction in an equally effective and time sensitive manner. FFPE blocks are available in most laboratory histology departments; therefore, the technique described here has the possibility of exponentially increasing the knowledge gained from these currently unused samples.

Disclosures

The authors disclose that there are no competing financial interests.

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