Capture and isolation of circulating tumor cells using photoacoustic flowmetry

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Summary

Circulating tumor cells (CTCs) are those cells that separate from a solid tumor and spread through the blood or lymphatic systems. While there are many open questions concerning the biology of CTCs, there is mounting evidence that some of these cells go on to create secondary tumors in distant organs, thus enabling metastatic disease. Detection of CTCs may have clinical impact by providing prognostic information. Furthermore, molecular and genetic analysis of CTCs may enable cancer biologists to answer questions about the metastatic process, such as whether these cells undergo epithelial-mesenchymal transition. Using a photoacoustic flowmeter, in which we induce ultrasonic responses from circulating melanoma cells (CMCs), we identify, capture, and isolate these cells for further analysis.

Keywords

diagnostic; metastasis; optical; optoacoustic; ultrasound

1 Introduction

CTCs are those cancer cells that separate from a tumor and move through the blood or lymph systems to create secondary tumors. The correlation of CTCs and disease state has been studied in melanoma, breast, prostate, urologic, and other cancers with mounting evidence that CTCs have strong prognostic value in advanced cancer patients.

Detecting CTCs has the potential to monitor therapy, detect relapse, and guide clinical management of cancer treatment and is an ongoing effort that has met with some success using immunocytochemical, immunomagnetic, microfluidic, and molecular methods. Though these methods are sensitive and have certain advantages, in all cases they require hours to perform and require the intervention of a skilled technician or cytologist to make a determination of the existence of a CTC. The photoacoustic method in this proposal is

objective and provides an immediate, sensitive, and unambiguous assay of CMCs in particular, due to their melanin content.

Photoacoustics is a process using light to create sound. In the biomedical sense, laser light is directed into cells or bulk tissue where it is selectively absorbed by structures of interest. We have performed CMC detection due to their natural light absorber, melanin, using our photoacoustic flow cytometer (Figure 1). The advantage of using photoacoustic detection over conventional flow cytometry is that cells can be specifically targeted with laser energy and the ultrasonic wave generated in these cells is strong enough for single cell detection. Additionally, cells in the blood sample remain in suspension under gentle flow conditions, giving high CTC viability for later analysis.

2 Materials

2.1 Blood Separation

- BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Heparin or a BD Vacutainer® Blood Collection Tube
- Histopaque®-1077 (not needed if you use the CPT (cell preparation tube).)
- Pasteur pipettes or 100–1000 ul pipette tips and pipettor
- 15 milliliter Falcon tube
- Plastic rack (for holding CPT)
- Blood drawing materials (butterfly syringes, gauze, band-aids, alcohol pads, tourniquet, and a person willing to take and give blood)

2.2 Photoacoustic Flowmetry

2.3 Capture and Isolation

Leica DMI4000B with Eppendorf Microinjection System: This is the piece of equipment known as the micromanipulator that allows us to grab single cells and place them in single wells.

2.4 Immunohistochemistry

- Solution of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)
- Solution of 10% normal goat serum in phosphate buffered saline (PBS)
- Phosphate buffered saline (PBS)
- Melan-A monoclonal primary antibody raised in rabbit (NBP1-30151, Novus Biologicals)
- CD-45 monoclonal primary antibody raised in mouse (NBP1-35553, Novus Biologicals)
- 16 well LabTek chamber slide coated with 1% poly-L-lysine for cell adhesion
- 0.1 ug/ml solution of DAPI in phosphate buffered saline (PBS)

3 Methods

3.1 Blood Separation

1. Obtain two 6 milliliter blood samples in CPTs or in vacutainer tubes. Use the second tube drawn for the melanoma test. This prevents contamination from skin cells. (If using CPT skip to step 4)
2. Place 3 milliliters of Histopaque 1077 in a 15 milliliter Falcon tube.
3. Carefully layer 6 milliliters of blood onto the histopaque layer. Do not allow the blood to break the surface of the histopaque layer.
4. If using CPTs invert tube 8–10 times to mix anticoagulant and blood together (do not shake).
5. Centrifuge the sample at 1800 g for 30 minutes.
6. At this point the blood should be separated into layers consisting of: Blood plasma on top, a white blood cell layer, a layer of histopaque or a polyester gel (if using CPT), and a layer of red blood cells on the bottom. Carefully pipette out half of the blood plasma above the white blood cell layer.
7. Pipet the white blood cell layer into a separate 15 milliliter Falcon tube and fill the tube with phosphate buffered saline.
8. Centrifuge the cells at 300 g for 15 minutes, pipet out excess fluid and dilute cells in 5 milliliters of phosphate buffered saline.

3.2 Photoacoustic Flowmetry

1. Allow a 1% bovine serum albumin solution to sit in the sample syringe, the tubing, and the flow chamber for 1 hour. This solution creates a hydrophobic barrier inside the tubing/syringe so that cells are less likely to stick during flow.
2. Turn on the laser system and let it warm up 15 minutes before testing.
3. Put the sample syringe pump on an angle using a doorstop or similar device. This helps keep the cells from sticking to the syringe.

4 Sample treatment

1. Perform whole blood separation protocol and RBC lysis buffer treatment.
2. When diluting the sample before running it through the system use mono-poly resolving medium as the dilutant. This assures very low settling velocity for the cells which prevents them from sticking to the flow path in the system.

5 Transducer alignment

1. Visually align the transducer element with the center portion of the quartz in the flow chamber.

2. Add acoustic gel to the transducer and use the XYZ mechanical stage to raise the transducer until it contacts the tape at the bottom of the flow chamber.
3. Fill the flow chamber with deionized water. This helps with viewing the cells in the flow chamber during testing as well as acoustic coupling for detection.
4. Place the .37 NA 1000 micrometer optical fiber 7.2 mm away from the front surface of the flow chamber. Please note that this is to assure the laser beam covers the entire chamber and the calculations will not work out correctly since the numerical aperture of our fiber is not exactly .37 due to the launching conditions so this 7.2 mm is an experimentally measured value.
5. Observe all standard laser safety protocols and get the laser ready to pulse.
6. Insert a stripped wire of 30 gauge size into the flow chamber where the irradiated beam should be.
7. Fire the laser continuously at 20 Hz.
8. The oscilloscope should show a signal somewhere in the time domain. At this point it is necessary to adjust the transducer in the z-axis by adjusting the XYZ mechanical stage until the initial photoacoustic response starts at 3.9 microseconds. The window for detection will be approximately between 3.9 microseconds and 4.9 microseconds so this is the time that should be set on the oscilloscope.
9. Remove the wire and purge the flow chamber with deionized water.

6 Detection electronics

1. The oscilloscope should be set to take 4 averages to reduce noise.
2. The RITEC system should be set on 32 dB amplification, with a 1 MHz high pass filter.

7 Running the system

1. Load a syringe full of mineral oil into the horizontal syringe pump.
2. Load a syringe full of sample into the slightly angled syringe pump.
3. Purge both pumps up to the connection point where the fluids combine before the flow chamber. (Purge means to hold down the button so that the fluid quickly flows to that location)
4. Run the syringe pumps at 20 microliters/minute.
5. Every two minutes the sample syringe must be rotated 180 degrees to prevent cell settling/sticking to the system.
6. Fire the laser on continuous at 20 Hz.
7. Look for cancer cell signals and save them when they occur.

8 Capturing the cells

1. Once a signal has been identified as having melanoma, a second engineer, who is watching the flow system with the microscope while wearing safety glasses, captures the cells using a small eppendorf tube at the end of the system. Once the cells are captured they are fluorescently labeled for analysis.

8.1 Capture and Isolation

8.2 Immunohistochemistry

1. Obtain single droplets containing cancer cells/white blood cells in a 16 well LabTek chamber slide
2. Allow cells to settle/adhere to surface for at least 30 minutes in a 5% CO₂ incubator.
3. Fill wells with Formalin solution (4% formaldehyde) and let sit for 15 minutes
4. Remove fixing solution and wash 3X with phosphate buffered saline (PBS) for 10 minutes each time.
5. Remove phosphate buffered saline (PBS) and incubate each slide with 10% goat serum in phosphate buffered saline (PBS) for 1 hour at room temperature.
6. Remove goat serum solution and add the primary antibody solution for 1 hour in a humidified chamber. Primary antibody solution is 1% CD-45 antibody and 1% Melan-A antibody in 10% goat serum in phosphate buffered saline (PBS).
7. Remove antibody solution and wash 3X with phosphate buffered saline (PBS) for 10 minutes each time.
8. Remove phosphate buffered saline and add fluorescent secondary antibodies to incubate for 1 hour in a dark humidified chamber. Secondary antibody solution is 0.05% Alexa fluor 488 goat anti-rabbit antibody and 0.05% Alexa fluor 546 goat anti-mouse antibody in 10% goat serum in phosphate buffered saline (PBS).
9. Remove antibody solution and wash 3X with phosphate buffered saline (PBS) for 10 minutes each time.
10. Remove the phosphate buffered saline (PBS) and add 0.1 ug/ml of DAPI and incubate for 1 minute.
11. Remove DAPI solution and wash 3X with phosphate buffered saline (PBS) for 10 minutes each time.
12. Directly view cells in phosphate buffered saline solution under microscope or mount cells using a resin-based mounting medium and seal the slide with a coverslip and nail polish.

Part I. Typical results—Typical good results showing staining between HS936 cells and white blood cells are shown below. The red fluorescence is staining the white blood cells and the green fluorescence is staining the HS936 cells. This image was taken by an overlay

of 3 separate images using the imaging software in the microscopy core. It is important to note that the correct filters must be inserted into the microscope to adequately take these images. If these results are not obtained due to low fluorescent intensity the concentration of primary and secondary antibodies can be increased to increase fluorescence. Additionally, if everything seems to have significant fluorescence then the antibodies can be lowered in concentration.

10 What to do if visible RBC contamination remains

1. Add a 2:1 ratio of RBC lysis buffer to cell solution. (Make sure the cells are mixed up so that the lysis buffer can lyse the red blood cells)
2. Incubate for 5 minutes on a shaking platform or with periodic inversions.
3. Centrifuge at 300 g for 15 minutes then remove supernatant carefully without disturbing the pellet.
4. Dilute cells into 5 milliliters of phosphate buffered saline. 5. If red blood cells are still apparent, repeat steps 1–4.

10.1 Troubleshooting

- Blood must be centrifuged within 2 hours of drawing.
- Blood must be 18–25 °C for correct centrifugation to occur. The density of the materials varies with temperature and if the temperature is not correct then the centrifugation will not be correct.
- CPTs require a minimum of 6 milliliters of blood for correct centrifugation to occur.
- CPTs require that the blood be centrifuged between 1500–1800 RCF (relative centrifugal force) which can be converted for any centrifuge by the following formula. $RPM = \sqrt{RCF \times (100,000) / (1.12 \times r)}$

where RPM stands for revolutions per minute, RCF stands for relative centrifugal force or “g-force” and r stands for radial distance from the end of the centrifuge tube while spinning from the center rotor axis of the centrifuge. This will change based upon the size and type of centrifuge.

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Fig. 1. The photoacoustic flowmeter uses laser delivered by an optical fiber to induce ultrasonic waves in targeted cells (such as circulating breast cancer cells). Cells that are identified as cancer cells are diverted for further analysis.