Ref C88801

8 Assays

CytoTrapNano™

Circulating Tumor Cell (CTC) Assay

For Research Use Only (RUO)

Instructions for Use

Version: April 30, 2019

INTENDED USE

The CytoTrapNano® CTC Assay is intended for the enumeration of circulating tumor cells (CTCs) of epithelial origin (CD45-, EpCAM+, and cytokeratin+) in whole blood.

For Research Use Only.

SUMMARY AND EXPLANATION

Circulating tumor cells (CTCs) are cells that have shed into the vasculature from a primary tumor and circulate in the bloodstream. CTCs thus constitute seeds for subsequent growth of additional tumors (metastasis) in vital distant organs, triggering a mechanism that is responsible for the vast majority of cancer-related deaths. CTCs thus could be considered a “liquid biopsy” which reveals metastasis in action and therefore provides live information about the disease status of a patient. Blood tests are easy and safe to perform and can be administered repeatedly during the course of disease progression. As disease progresses in individual patients, analysis of blood samples shows a propensity for increased CTC detection. CytoTrapNano® technologies with the requisite sensitivity and reproducibility to detect and enumerate CTCs in patients with metastatic disease have been developed. The CytoTrapNano® Chip Holder is designed for use with the CytoTrapNano® Circulating Tumor Cell Kit (CytoTrapNano® CTC Kit) and CytoTrapNano® Chips and Covers. After CTCs are immobilized by the CytoTrapNano® CTC Kit, Images are captured with a semi-automated fluorescence microscope. Analysis and enumeration of CTCs can be done manually by a pathologist or certified reviewer using the given guidelines.

WORKFLOW OF THE PROCEDURE

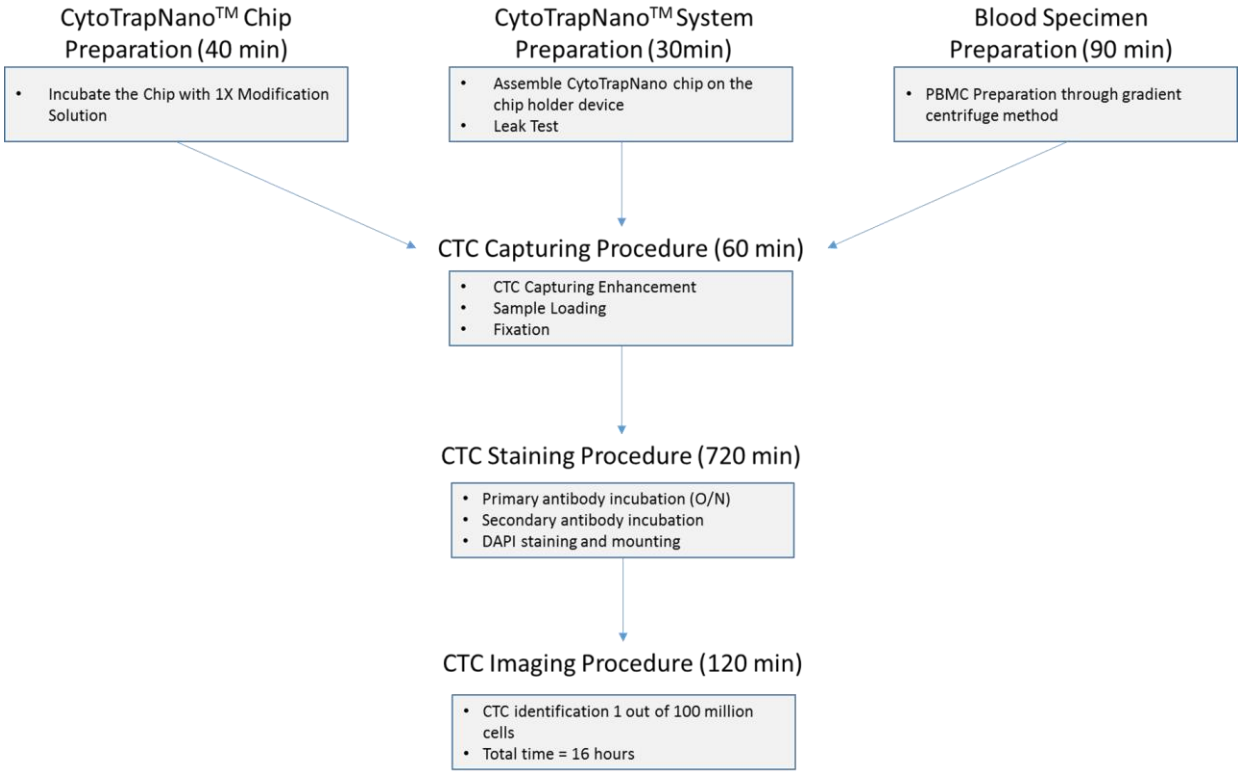

Figure 1. Workflow for performing a CytoTrapNano® assay.

MATERIALS PROVIDED (1 KIT)

Primary Box: Store at 2-8 ºC

- **Instructions for Use**
- **8 x CytoTrapNano® Chip**, store at 2-8 ºC
- **8 x PDMS Substrate Cover**, store ate 2-8 ºC
- **2 x 12mL Enrichment Solution:** Contains 100% enrichment ingredients, store at 2-8 ºC
- **0.120mL 10x Fixative:** Contains 20% fixative ingredients, store at 2-8 ºC

Inner Box: Store at -20 ºC

- **1 x 0.096mL 50x Blocking Solution:** Contains 100% blocking ingredients, store at -20 ºC
- **1 x 0.020mL 100x Permeabilization Solution:** Contains 10% permeabilization ingredients, store at -20 ºC
- **8 x 25ug 40x Modification Solution:** Contains 0.5% modification ingredients, store at -20 ºC
- **0.058mL Capture Enhancement Solution:** Contains 0.03% cell capture ingredients, store at -20 ºC
- **0.030mL Staining Solution:** Contains 1.5% staining ingredients, store at -20 ºC

- **0.008mL Fluorescent Dye Solution 1:** Contains 0.1% fluorescent dye ingredients, store at -20 ºC
- **0.008mL Fluorescent Dye Solution 2:** Contains 0.1% fluorescent dye ingredients, store at -20 ºC
- **0.008mL DAPI Stain:** Contains 0.005% 4', 6-diamidino-2-phenylindole, dihydrochloride, store at -20 ºC
- **0.500mL Mounting Solution:** store at -20 ºC

MATERIALS AND EQUIPMENT REQUIRED, NOT PROVIDED

- Blood Drawing Kit
- CytoTrapNano® Chip Holder
- CytoTrapNano® Chip (dummy chip)
- Fluorescent Microscope (e.g. Nikon Eclipse Ti2-E Inverted Microscope System)
- 1.5 mL Centrifuge Tube
- 15 mL Centrifuge Tube
- 200µL tip and pipette (Gilson # F123601 PIPETMAN P200, 50–200µL), or equivalent
- 1000µL tip and pipette (Gilson # F123602 PIPETMAN P200, 200–1000µL), or equivalent
- Test tube racks
- 60 mm Petri dishes or equivalent container
- Double sided tape (CMC Converting, NITTO #5600, customized 20mm wide strip)
- Kim Wipe (Kimberly-Clark Corporation 34155), or equivalent
- Horizontal swing out style rotor (i.e. swing bucket) centrifuge capable of 800 x g
- Vortex mixer (Fisher Scientific, cat# 02-215-365, or equivalent)
- Cover glass (cat# 12-544-A, 22x30-1.5, Fisher Scientific, or equivalent)
- Aspiration system (aspirator) (Med Supply Partners, cat# RG-EV432, or equivalent)
- Shaker (Nutator) (Benchmark Scientific, cat# M2100, or equivalent)
- Standard glass slide (cat#:12-544-1, Fisher Scientific, or equivalent)
- 1 pouch PBS powder for making 1L PBS (Sigma-Aldrich, cat# P-5368, or equivalent)
- 10mL Serological Pipette (Sigma Aldrich, cat# CLS4488, or equivalent)
- 4°C Centrifuge (ThermoFisher Scientific, cat# 75004381, or equivalent)
- 8 x SepMate 15mL Conical Tubes (Stemcell Technologies, cat# 85415)

WARNINGS AND PRECAUTIONS

1. For in Research Use Only.
2. Please read the entire contents of these Instructions for Use before testing samples.
3. Caution: Collect blood using CytoTrapNano™ Blood Drawing Kit only. CTC's are fragile and require preservation for accurate analysis.
4. Caution: All personnel should follow universal precautions and use laboratory safety equipment (i.e., safety glasses, laboratory coat, gloves).
5. Caution: Microbial contamination of reagents can cause erroneous results and should be avoided.

6. **Warning:** All biological specimens, chips, PDMS substrate cover and other materials coming into contact with the specimen(s) are considered biohazardous. Handle as if capable of transmitting infection. Treat and dispose of waste using proper precautions and in accordance with local, state, and federal regulations. Never pipette by mouth.
7. **Warning:** Some of the reagents contain sodium azide as a preservative. If swallowed, seek medical advice immediately. Keep out of reach of children. Keep away from food and drink. Wear suitable protective clothing. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop. Risk and safety phrases R22 (Harmful if swallowed) and S28 (After contact with skin, wash with plenty of soap).
8. **Warning:** Some of the reagents contain Triton® X-100 as a preservative. Symptoms of overexposure to Triton® X-100 may include eye burns and skin irritation as well as lung damage if aspirated. CAUSES EYE BURNS.
9. Operator training is required to perform the test procedure.

REAGENT STORAGE AND HANDLING

NOTE: This kit is shipped with ice packs, please store the "Box -20C" at -20ºC immediately after arrival and the other components at 2-8ºC.

TEST PROCEDURE (CytoTrapNano™ Chip Processing for CTC Enumeration)

A. CytoTrapNano™ chip preparation and modification

1. Prepare 1XPBS by mixing the powder included in the PBS pouch with 1 L of distilled or deionized water (reagent-quality water). Dissolve completely for at least 20 minutes. Note: 1xPBS can be stored at 2-8ºC for up to 6 months.
2. Thaw 40x Modification Solution at room temperature and quick-spin for 5 seconds.
3. Make 1x Modification Solution by adding 200 µL of 1x PBS into a tube of 25µg 40x Modification Solution, vortex to mix the solution.
4. Wash each of the 2 CytoTrapNano™ Chips in the Petri dishes 3 times with 5mL of 1xPBS.
5. Remove PBS from the CytoTrapNano™ Chip surface and surrounding Petri dish with aspirator.
6. Spread 195-200 µL 1x Modification Solution (of the ~400 µL) all over the channels on each of the CytoTrapNano™ Chip(s) to be used (still in the Petri dishes) note: avoid air bubbles at this step.
7. Incubate the CytoTrapNano™ Chip(s) at room temperature for 35±5minutes.
8. Wash each CytoTrapNano™ Chip in the Petri dish 3 times with 5mL 1xPBS.
9. Remove the PBS from each CytoTrapNano™ Chip surface and Petri dish with aspirator. Air dry the chip(s).

NOTE: Do not touch the channels of CytoTrapNano™ Chip surface when aspirating.

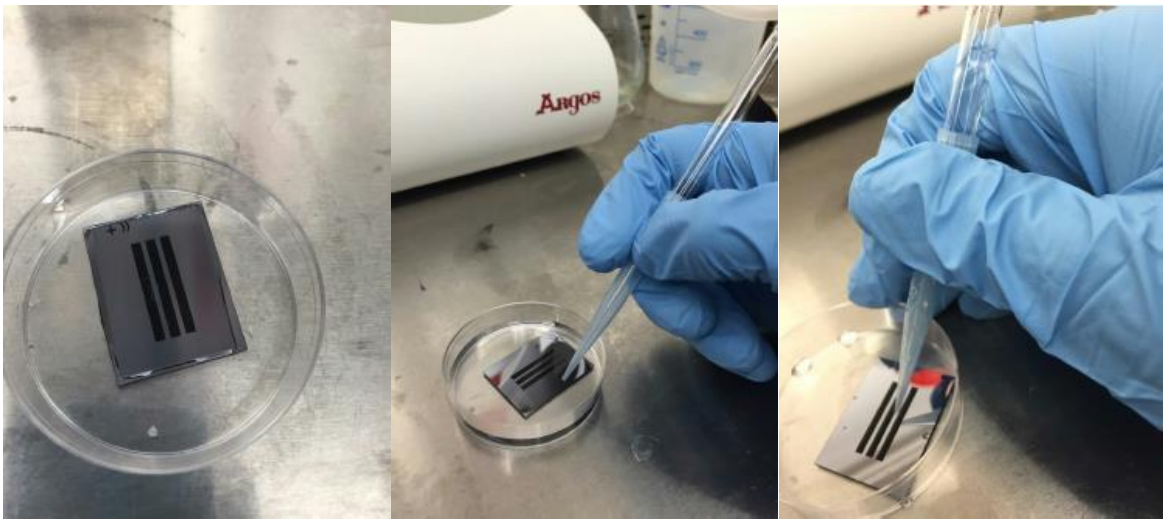

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Figure 2. CytoTrapNano™ Chip surface modification showing correct and incorrect position of solution aspiration.

B. Initial blood specimen processing

Note: Use 2 mL of blood sample for 1 CytoTrapNano™ Chip.

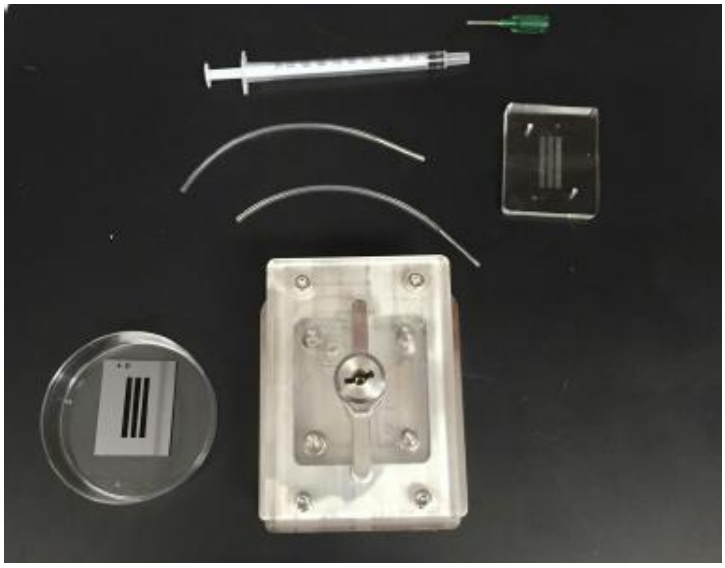
1. Collect 1 tube of fasting venous blood specimen (9.5mL > total volume > 3.5mL) through standard phlebotomy procedure using 1 ACD-containing yellow-top vacutainer. If the sample cannot be processed immediately, store in 4 °C for up to 24 hours.
2. Recover the blood sample, 1x PBS, and Enrichment Solution to room temperature.
3. Label one 15mL SepMate conical tube and add 6mL Enrichment solution. Take care not to create bubbles.
4. Using a 10mL serological pipette, remove the blood sample and transfer it to another 50mL centrifuge tube. Measure the volume of blood in the serological pipette, and/or in the new tube. *Make note of the volume measured*.
5. Add 1XPBS to the new tube in a volume equal to 3mL less than the volume measured. (e.g., if 8mL of sample are measured, add 5mL 1XPBS. If 4mL of sample is measured, add 1mL PBS). Mix by pipetting gently. Put the 1xPBS in 4ºC for the rest of the procedure.
   Note: This will always result in a 1:1 dilution of blood to non-blood.
6. SLOWLY and GENTLY add 4.0mL of the mixture from step B5 to the top of the Enrichment solution in the SepMate tube.
7. Centrifuge with the following settings: 10 minutes, 1200 x g, Room Temperature, with acceleration level 9/deceleration level 9.
8. Make sure that the Peripheral Blood Mononuclear Cell (PBMC) layer appears as a clearly distinct layer between the Enrichment solution and plasma.
9. Pour the PBMC layer into a new, labeled, 15mL centrifuge tube (not Sep-Mate tube).
   Note: In order to avoid red blood cell contamination when pouring, do not hold the tube upside down for more than 2 seconds.

10. Centrifuge with the settings: 10 minutes, 300 x g, 4°C, acceleration level 9/ deceleration level 9.
11. Discard the supernatant.
12. Add 1mL cold 1X PBS to pellet, resuspend gently. Add 4mL cold 1X PBS.
13. Centrifuge: 10 minutes, 300 x g, 4°C, acceleration level 9/ deceleration level 9.
14. Discard the supernatant.

C. Capture Enhancement and Preparation

1. Add a 4 µL aliquot of Capture Enhancement Solution to 196µL 1X PBS. Mix by pipetting.
2. Add the 200µL diluted Capture Enhancement Solution (from step C1) to resuspend the pellet, mix by pipetting.
3. Incubate at room temperature, on a nutator (gently) for 30 minutes (Suggestion: preform part D during this period).
4. Add 3mL cold 1X PBS, invert slowly and gently 5 times.
5. Centrifuge with the following settings: 300 x g, 10 minutes, 4°C, acceleration level 9/deceleration level 9.
6. Aspirate the supernatant. NOTE: If no aspiration system is available, place the tube upside down on absorbent paper (e.g., a Kimwipe) until it dries (no more than 5 minutes).
7. Bring the volume up to 200µL with 1X PBS. Gently resuspend pellet.
8. Process on chips as soon as possible.

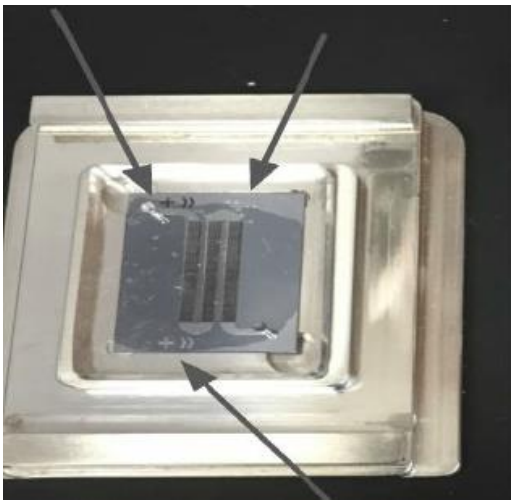
D. Device Assembly and Leak Test

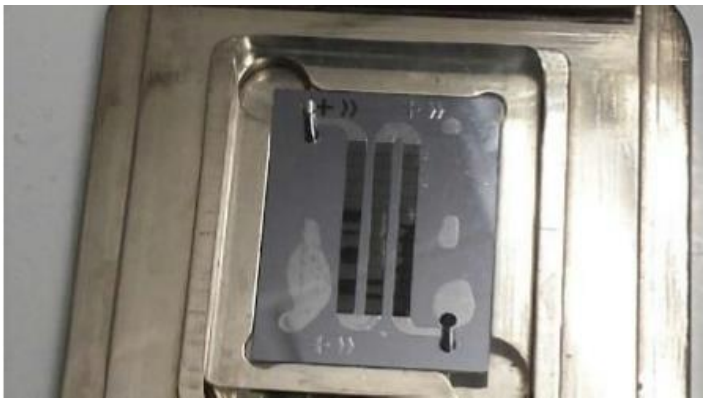


1. Sterilize the CytoTrapNano® Chip holder that is going to be used with 70% ethanol using Kimwipe or equivalent material.
2. Dry the CytoTrapNano® Chip holder by Kim Wipe or air-dry for 5 minutes.
3. Place the MS-modified Chip onto the CytoTrapNano® Chip holder.

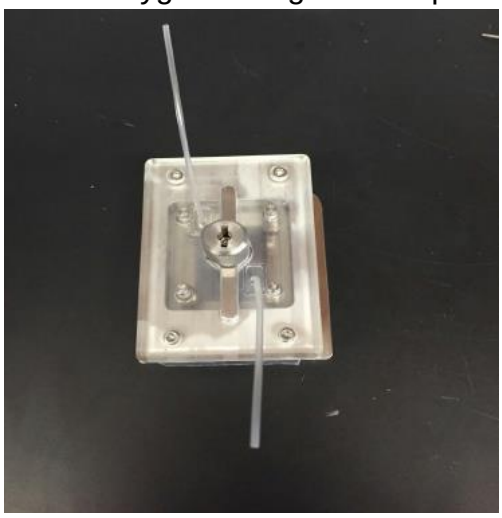
4. Open the package of a PDMS substrate cover, and use the scotch tape to clean the channel side surface several times.

NOTE: Do not touch the PDMS substrate cover channel side surface with gloves or hands; dusty environment should be avoided.





5. Place the PDMS substrate cover onto the CytoTrapNano® Chip with the channels and arrows/alignment markers aligned.
6. Close the CytoTrapNano® Chip holder and turn the knob until a light resistance is felt.
7. Insert the Tygon tubing with coupler into the inlet and outlet of the device.



8. Insert 18G needle onto a 1 mL syringe and fill the 1 mL syringe with at least 300 µL 1x PBS; connect the inlet tubing to the 1 mL syringe.
9. Place the 1 mL syringe onto a syringe pump, and run leak test with the flow rate of 1 mL/h.
10. The leak test is completed when the 1x PBS comes out of the outlet tubing with the same flow rate as it enters the inlet tubing.
11. The device is now ready for sample loading.

E. Sample Loading and Fixation

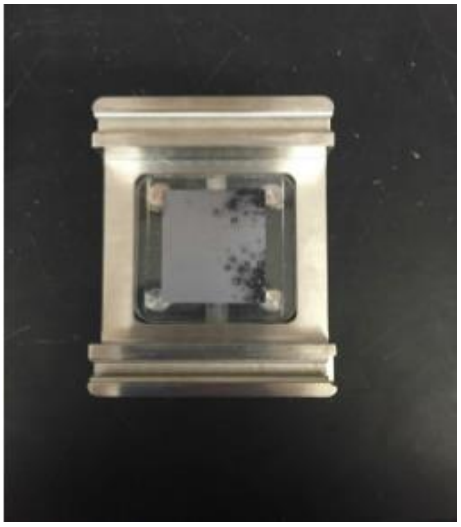
1. Disconnect the syringe from the inlet tubing, unplug the syringe and use a pipette to load the cell suspension into the syringe.

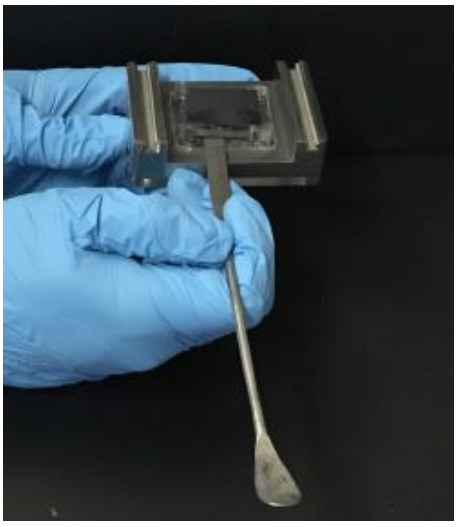
NOTE: Keep the syringe parallel to the ground during the procedure, and slowly roll the syringe while re-plugging and pushing the syringe until the cell suspension reaches the needle.

2. Place the syringe back to the syringe pump and reconnect the syringe with the inlet tubing.
3. Run the sample with the following settings: volume 220 µL, flow rate 0.5 mL/h. (check sample after 20 min, STOP if no fluid come out).
4. Prepare 1x Fixative (FX) with 1x PBS by adding 20 µL 10x Fixative into 180 µL 1x PBS and mix by pipetting, load 200 µL 1x Fixative into another syringe.
5. Stop the syringe pump when all the blood sample comes out of the needle, and reconnect to prepared syringe filled with 200 µL 1x Fixative, then run the 1x FX with the following setting: volume 200 µL, flow rate 0.5 mL/h.

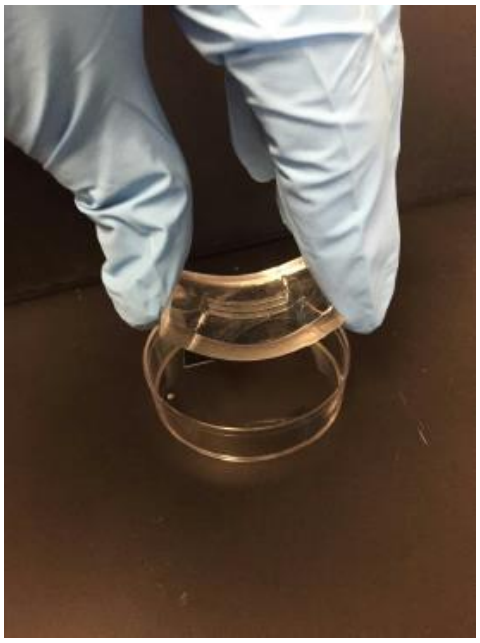
NOTE: Reconnect the tubing and restart the syringe pump ASAP.

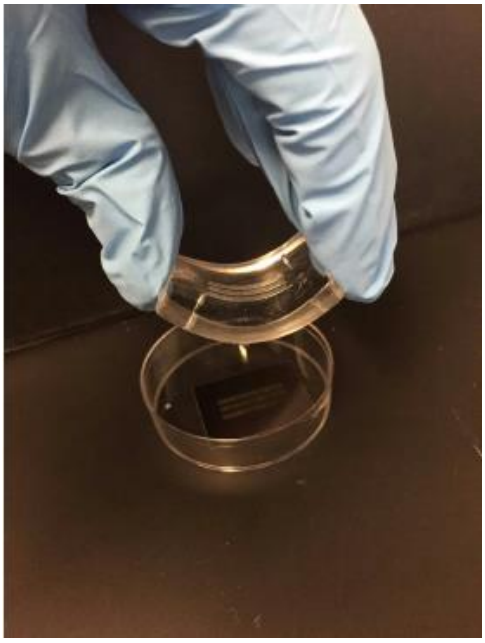
6. Stop the syringe pump and disconnect all the tubings.
7. Turn the knob and open the chip holder.
8. Take off the PDMS Substrate Cover with Chip from the top piece of the chip holder together.





9. Bend the PDMS Substrate Cover and release the Chip into a petri dish with a small volume (about 1 mL) of 1x PBS. Proceed to primary antibody staining or store at 4 °C.





F. Primary Staining <u>for Each Chip</u>

1. For each chip, mix 1µL of 200X Permeabilization Solution with 199µL 1XPBS to make 1X Permeabilization Solution.
2. Apply 200µL 1X Permeabilization Solution to the chip surface, **carefully** cover the 3 channels completely (see figure 9). Wait 10 minutes at room-temperature.
3. For each chip, mix 4µL of 50X Blocking Solution with 196µL 1XPBS to make 1X Blocking Solution.
4. Make the Primary Staining Solution by mixing 200µL 1x Blocking Solution with 3 µL Staining Solution. Mix the solution by vortexing and keep on ice before use.
5. Rinse the chip with 5mL 1XPBS three times.
6. Apply 200 µL of the Primary Staining Solution onto the chip surface and spread to completely cover the 3 channels.
7. Place the chip into a humidified chamber such as a slide box with wet paper towel and incubate at 4°C overnight.
8. Proceed to Part G.

Figure 9. Chip with staining solution applied.

G. Secondary staining <u>for Each Chip</u>

1. Take the CytoTrapNano™ chip previously stained with the primary antibody out of the 4ºC, and allow the chip to equilibrate to room temperature for 30 to 60 minutes.
2. Make 1X Blocking Solution by mixing 5µL 50X Blocking solution with 245µL 1XPBS
3. Gently wash the chip with 5 mL 1XPBS three times.
4. Make the secondary staining solution by mixing 250 µL 1X Blocking Solution with 0.5µL Fluorescent Dye Solution 1 and 0.5µL Fluorescent Dye Solution 2. Vortex the solution and shield the solution from light.
5. Apply 200µL secondary staining solution onto the chip surface and spread to completely cover the 3 channels.
6. Keep the chip in the dark for 45 minutes at room temperature, as the dyes are light sensitive.
7. Label a microscope slide with the sample name and number.
8. Wash the chip with 5mL1XPBS three times and dry with aspirator.

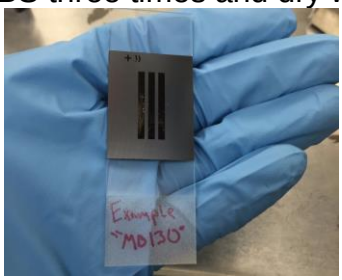


Figure 10. A chip mounted on a slide.

9. Prepare DAPI and Mounting solution by taking them out of the -20C freezer and letting them equilibrate to room temperature.
10. Dilute 0.5µL DAPI into 500µL PBS. Mix by vortex and make sure to shield the solution from light.

11. Apply 200µL 1:1000 DAPI to the chip surface and spread to completely cover the 3 channels.
12. Let the DAPI sit for 10 minutes, then rinse the chip with ~5mL 1XPBS once.
13. Apply double sided tape to the slide. Dry the bottom of the chip with a Kim wipe and attach it to the tape on the glass slide.
14. Apply 60µL of mounting solution onto the chip surface and spread to completely cover the 3 channels. Avoid any bubbles.
15. Place a 22mm x 30mm cover glass onto the chip. Avoid formation of bubbles.

NOTE: Avoid generating bubbles in the above steps; if there are bubbles, put the whole slide into PBS immediately and wait until the cover glass comes off, then repeat steps G11 to G15.

16. Lay the glass slide into the black box and let sit for one hour before scanning the sample. If the scan cannot be done in one hour then store the box at 4 ^{o}C.

Figure 11. Store chip in dark box.

H. Image Scanning

1. Load slides into the slide holder

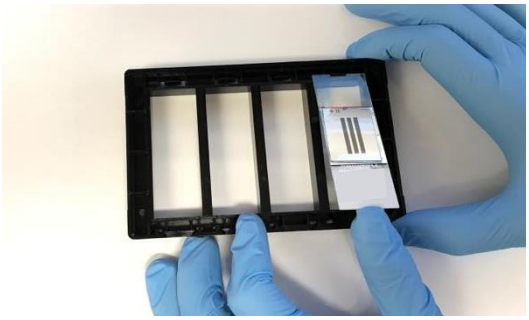


Figure 12. When a slide is inserted into the slide holder, the chip should face up (note: the two corners of the holder margin are indented to match the clips on the microscope carrier).

2. Place the loaded chip holder into the microscope, with the chip(s) facing down
3. Start Nikon Elements imaging software (version 4.6)
4. Click Play (shown in Figure 14)
5. Click 10x DAPI (shown in Figure 14)
    a. You can check the location of the light on the chip to adjust your location to one of the channels before focusing.

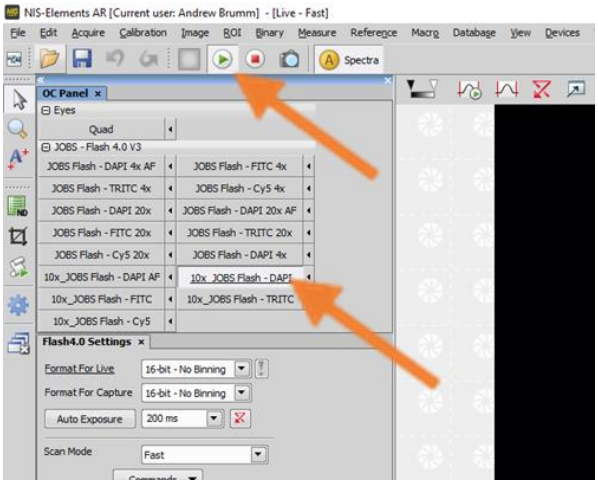


Figure 14. Nikon Elements software screen showing the location of the Play button and 10x DAPI button

6. Adjust contrast by changing the values shown below (Figure 15)
    a. Adjust the value in LUTS top right corner to show the cells best. Generally, between 300 and 1500
    b. In the top left corner of the LUTS tab, set the value to ~200 (the middle of the peak)
    c. The exposure should be around 100-150 for DAPI channel
7. Adjust the focus until the image is clear, like in Figure 15.

8. Repeat step 6 for the FITC and Cy5 channels using the “10x JOBS Flash -FITC” and “10x JOBS Flash - Cy5” buttons
    a. For FITC and Cy5, use about 200-300 for auto-exposure time
    b. For LUTS, follow the same process as DAPI
        i. FITC and Cy5 upper bounds ~1000 or lower

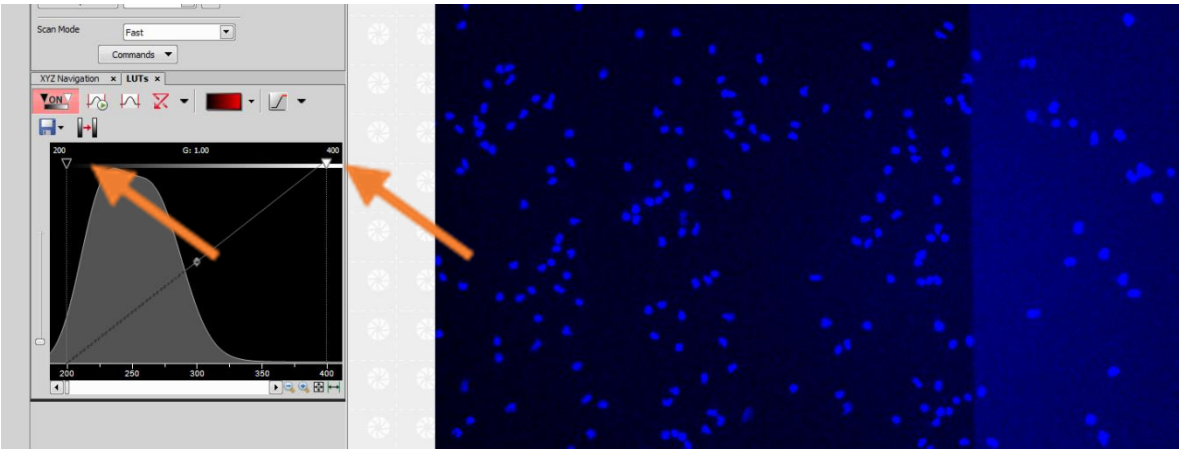


Figure 15. Adjusting the contrast

9. Click CytoLumina-4 chip scan 10x (in the “Run” section of the “JOBS Explorer” tab in bottom right-hand corner)
10. Press play (run job)
11. Select the fluorescent channels (DAPI, FITC, Cy5) in the “Select Channels” tab of the pop-up window, and select the area of the chip to scan by clicking and dragging on the main image so all 3 of the chip’s channels are covered by a box.

Figure 16. Select Channels

12. In the pop-up window select the well (of the slide holder) that your chip is in by hitting **ctrl + right click**
    a. Can adjust the colors based on preferences
13. Go to the “Scan large Image” tab of the pop-up window, then adjust the fields to 12x4
14. Press Play (Run), the screen should show that it is performing autofocus
15. Press Play, once told it is ready
16. Select focus points in the pop-up window. Use the main image to view the points first.
    a. Adjust the contrast for visibility (i.e. 100-170, but can vary)
    b. 9-15 focus points are standard (3-5 focus points per channel)
    c. Can move positions by right clicking on either the larger or smaller image, or by using the joystick
    d. Guidelines for point selection
        i. Avoid blots, dust and lines
        ii. Set near nicely scattered cells

iii. The autofocus depends on the strongest relative contrast
iv. It scans 200um (+/- 100 of the focus point) in the Z-axis

17. Press Point Names
18. When done, press Continue
19. Adjust the contrast until you can see all nanofiber channels on the chip
20. Select all the nanofiber channels, then click Finish to initiate the scan

I. Interpretation

Imaging parameters

- 10X objective
- DAPI (corresponding to nuclear staining) exposure time: 40-100 ms (Minimize the number of over-exposure events)
- FITC (corresponding to CK staining) exposure time: 200 ms
- TRITC (corresponding to CD45 staining) exposure time: 200 ms

Interpretation Dictionary

- DAPI is blue, and stains for nuclei
- TRITC is orange/red and stains for CD45
- FITC is green, and stains for cytokeratin
- CTCs are Cd45-/Cytokeratin+
- WBCS* are CD45+/Cytokeratin-
- *Granulocytes may be Cytokeratin+, but with a lobulated (kidney shaped) nuclei
- Cells meeting all the major criteria, and none of the exclusionary criteria are deemed “typical CTCs”
- Cells meeting all inclusion criteria but some exclusion (or suggestive exclusion) are deemed “atypical CTCs” and do not count toward CTC count.

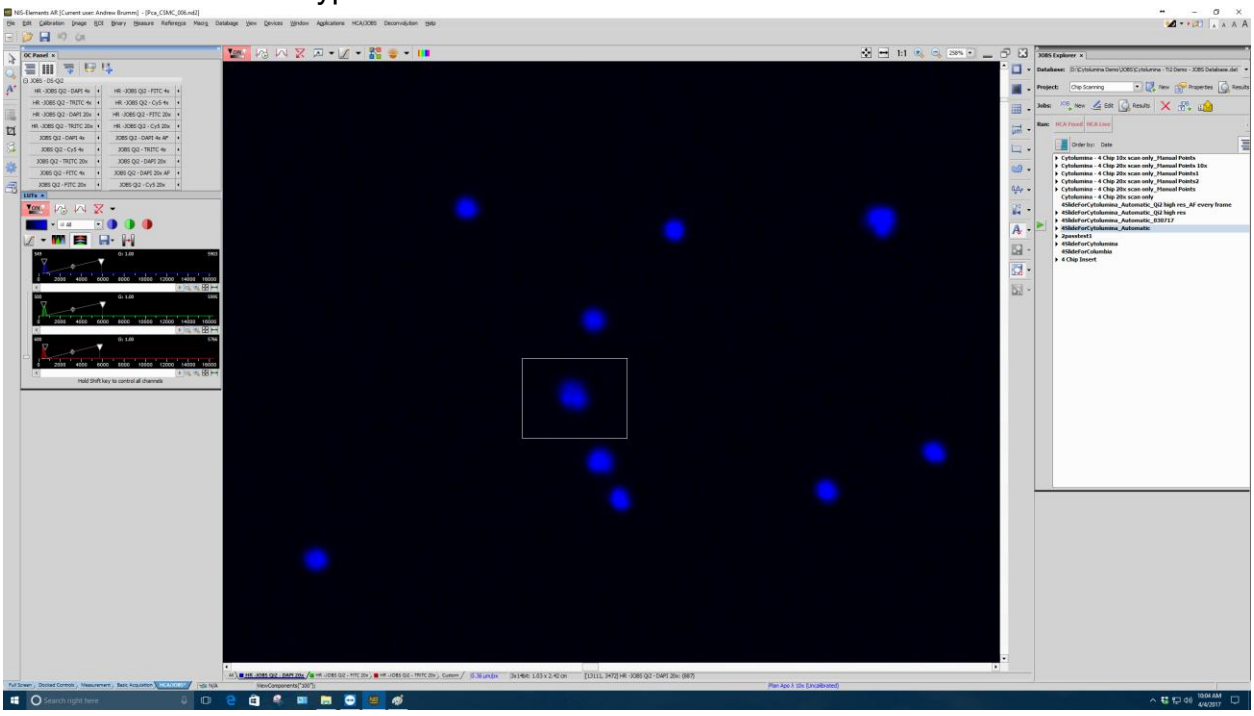


Figure 16. (Above) Image of cells showing the DAPI channel.
The DAPI channel shows the nuclei of the captured cells. CTCs will be DAPI+ and have a long diameter of at least 3um.

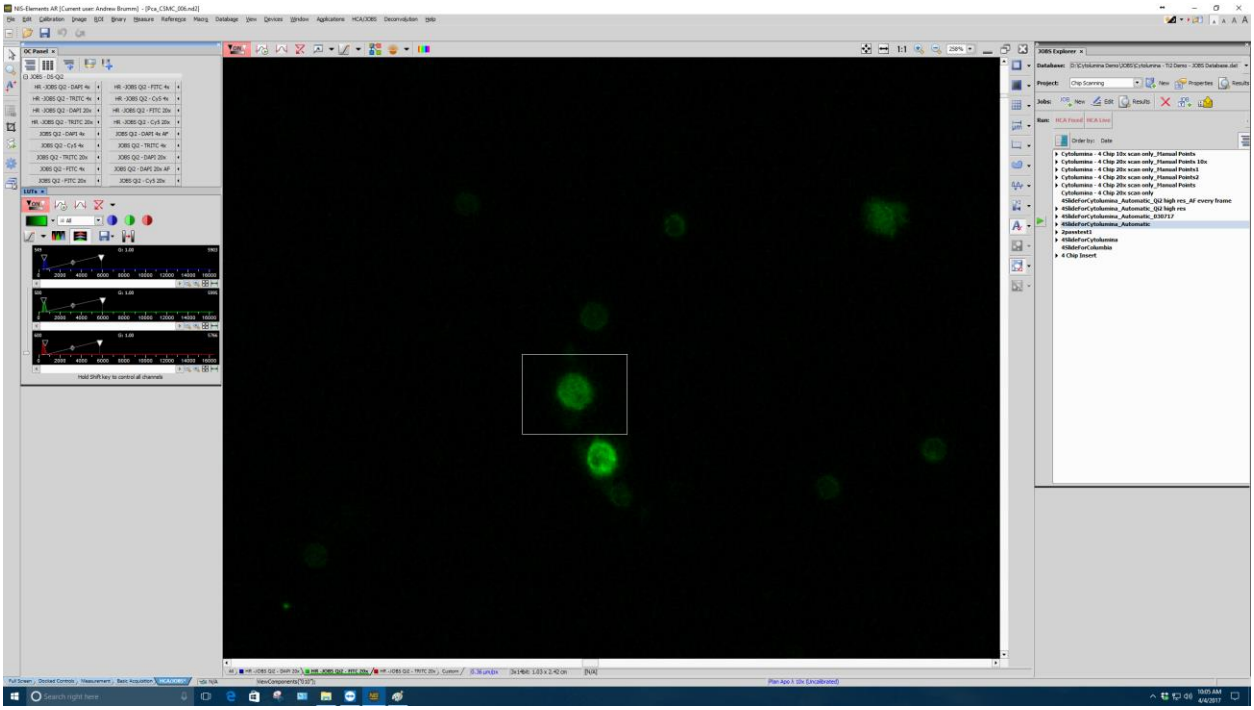


Figure 17. (Above) Image of cells showing the FITC channel.
The FITC channel stains for Cytokeratins. CTCs will be FITC+ and should have a nuclear shadow that is not lobular/kidney shaped.

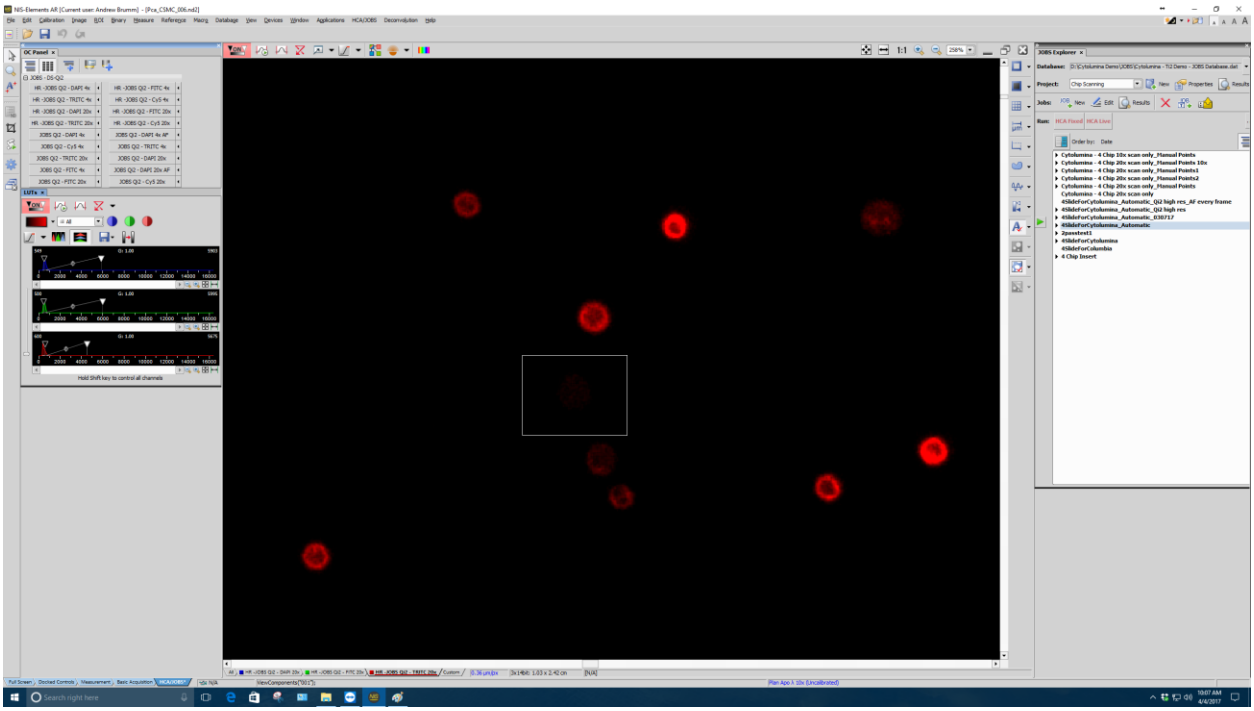


Figure 18. Image of cells showing the Cy5 channel.
The Cy5 channel stains for CD45. CTCs will be Cy5- and should not appear red. (compared to the background)

Major Criteria

Inclusion criteria (must have ALL)

- DAPI positive (2 or more times to surrounding background signal) without over-exposure
- Longest diameter larger than 3 um for nucleus in DAPI channel
- FITC positive (3 or more times to surrounding background signal) with nuclear shadow

Exclusion criteria (must NOT have ANY)

- TRITC positive (3 or more times to surrounding background signal), or
- Not applied to the dust or antibody aggregates attached to the target cell
- Weak TRITC signal with membrane or cytoplasm staining pattern, or
- Multi-lobular or kidney-shaped nucleus in DAPI channel, or
- Unclear nuclear morphology in DAPI channel but multi-lobular or kidney-shaped nuclear shadow was seen in FITC or TRITC channel, or
- Unclear nuclear morphology in DAPI channel due to cell clustering, or
- Ruptured nucleus in DAPI channel

Minor Criteria

Minor criteria suggestive of CTCs (for manual analysis)

- Nuclear-to-cell ratio (as defined by nuclear area [DAPI area] divided by cellular area [entire footprint including DAPI and FITC area]) 0.40 - 0.95
- No nuclear cleavage/groove in DAPI channel
- Presence of nucleoli as round or oval shadows within nucleus in DAPI channel
- The TRITC intensity of the target cell lower than the that of the surrounding cells

Minor criteria suggestive of non-CTCs (for manual analysis)

- FITC intensity close to saturation
- Presence of lobulated nuclear structure at z-axis

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