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Ovarian Cancer Detection Using Photoacoustic Flow Cytometry

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Ira A. Fulton Schools of Engineering
Arizona State University

July 16, 2019

Dear Dr. Steindel,

My coauthors and I wish to submit a revision for our manuscript entitled "Ovarian Cancer Detection Using Photoacoustic Flow Cytometry" for consideration by the JoVE. The authors have seen and approved the manuscript, including: Joel F. Lusk, Christopher Miranda, and Barbara S. Smith (corresponding author). We are glad to submit revisions for this invited paper to JoVE.

In this work, we explore a new application in photoacoustic imaging, towards promoting improved diagnostics for ovarian cancer metastasis. This is significant because 85-90% of patients will experience disease recurrence, however, there is no accurate method for detecting ovarian CTCs at the point-of-care. Thus, leading to limitations in clinical applications for the early detection of ovarian cancer metastasis. Photoacoustic detection is widely used for biomedical imaging applications, as presented in the author's previously published work. Recent studies have shown that folic acid functionalization is an effective strategy for targeting ovarian cancer cells by photoacoustics, however, no study to date has attempted to identify ovarian circulating tumor cells in flow. In this paper, we report on the development and testing of folic acid functionalized copper sulfide nanoparticles (FA CuS NPs) as photoacoustic contrast agents for the identification of ovarian circulating tumor cells in flow. Our results show the detection of ovarian CTCs in flow, down to 1 cell/ μL.

A response to reviewers has been included in the resubmission along with a revised manuscript. Each of the reviewer's comments were taken into consideration, and updates were made accordingly. The authors believe that these updates have significantly improved the manuscript quality. This manuscript is specifically being sent to JoVE due to the interest of its readership, who are highly in tune with advancements within the field of photoacoustic imaging and advanced diagnostic detection. This paper is relevant to readers in the areas of photacoustics, photacoustic flow cytometry, copper sulfide nanoparticles, circulating tumor cells, ovarian cancer, folic acid, and optoacoustic.

Thank you for your invitation and consideration of our manuscript.

Sincerely,

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TITLE:

Ovarian Cancer Detection Using Photoacoustic Flow Cytometry

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KEYWORDS:
photoacoustic, photoacoustic flow cytometry, ovarian circulating tumor cells, copper sulfide nanoparticles, folic acid, optoacoustic

SUMMARY:
A protocol is presented to detect circulating ovarian tumor cells utilizing a custom-made photoacoustic flow system and targeted folic acid-capped copper sulfide nanoparticles.

ABSTRACT:
Many studies suggest that the enumeration of circulating tumor cells (CTCs) may show promise as a prognostic tool for ovarian cancer. Current strategies for the detection of CTCs include flow cytometry, microfluidic devices, and real-time polymerase chain reaction (RT-PCR). Despite recent advances, methods for the detection of early ovarian cancer metastasis still lack the sensitivity and specificity required for clinical translation. Here, a novel method is presented for the detection of ovarian circulating tumor cells by photoacoustic flow cytometry (PAFC) utilizing a custom three dimensional (3D) printed system, including a flow chamber and syringe pump. This method utilizes folic acid-capped copper sulfide nanoparticles (FA-CuS NPs) to target SKOV-3 ovarian cancer cells by PAFC. This work demonstrates the affinity of these contrast agents for ovarian cancer cells. The results show NP characterization, PAFC detection, and NP uptake by fluorescence microscopy, thus demonstrating the potential of this novel system to detect ovarian CTCs at physiologically relevant concentrations.

INTRODUCTION:
Ovarian cancer is one of the deadliest gynecological malignancies and resulted in an estimated 184,800 deaths worldwide in 2018[1]. Multiple studies have shown the correlation between ovarian cancer progression (i.e., metastasis) and the presence of CTCs[2–4]. The most common method for detection and isolation of CTCs utilizes the Cellsearch system, which targets the EpCam receptor[5]. EpCam expression, however, is downregulated in epithelial to mesenchymal

transition, which has been implicated in cancer metastasis[6]. Despite advances, current clinical technologies still suffer from low accuracy, high cost, and complexity. Due to these drawbacks, new technologies for the discovery and enumeration of ovarian CTCs has become an important area for research.

Recently, PAFC emerged as an effective method for the noninvasive detection of cancer cells, analysis of nanomaterials, and identification of bacteria[7–9]. PAFC differs from traditional fluorescence flow cytometry by detecting analytes in flow by utilizing photoacoustics. The photoacoustic effect is generated when laser light is absorbed by a material that causes thermoelastic expansion, producing an acoustic wave that can be detected by an ultrasound transducer[10-11]. Advantages of PAFC over traditional flow cytometry methods include simplicity, ease of translation to clinical settings, and the detection of CTCs at unprecedented depths in patient samples[12,13]. Recent studies have utilized PAFC systems for the detection of cells using endogenous and exogenous contrast[14,15]. Near infrared (NIR) light-absorbing contrast agents such as indocyanine green dye, and metal NPs (e.g., gold and CuS) have been used for the selective labeling of cells and tissues in combination with photoacoustic imaging[16–18]. Due to the improved penetration depth of NIR light within biological tissues, photoacoustic detection of absorbers can be performed at greater depths for clinical applications. Because of its great potential for use in the clinic, the combination of targeted NIR contrast agents with PAFC has generated considerable interest for the detection of CTCs.

PAFC in combination with targeted contrast agents provides an improved approach for high-throughput analysis of patient samples with enhanced accuracy and targeted detection of CTCs. One of the principal detection strategies for CTCs is the specific targeting of membrane proteins present on the cell of interest. One notable characteristic of ovarian CTCs is the overexpression of folate receptors located on their outer membrane[19]. Folate receptor targeting is an ideal strategy for the identification of ovarian CTCs in blood because endogenous cells, which have higher expression of folic acid receptors, are generally luminal and have limited exposure to the bloodstream[20]. Copper sulfide NPs (CuS NPs) have recently been recognized for their ability to target folate receptors expressed on cancerous cells[21]. Combined with their biocompatibility, ease of synthesis, and absorption deep in the NIR, these NP contrast agents make an ideal targeting strategy for the detection of ovarian CTCs utilizing PAFC.

This work describes the preparation of FA-CuS NPs and their use for the detection of ovarian cancer cells in a photoacoustic flow system. CuS NPs are modified with folic acid to specifically target ovarian CTCs and emit a photoacoustic signal when stimulated with a 1,053 nm laser. The results indicate the successful detection of ovarian cancer cells incubated with these photoacoustic contrast agents within the PAFC system. These results show detection of ovarian cancer cells down to concentrations of 1 cell/µL, and fluorescence microscopy confirms successful uptake of these particles by SKOV-3 ovarian cancer cells[22]. This work provides a detailed description of the FA-CuS NPs synthesis, preparation of samples for fluorescence microscopy, construction of the photoacoustic flow system, and the photoacoustic detection of ovarian cancer cells. The presented method shows successful identification of ovarian CTCs in flow utilizing FA-CuS NPs. Future work will focus on the clinical application of this technology

towards the early detection of ovarian cancer metastasis.

PROTOCOL:

1. Nanoparticle synthesis and functionalization

NOTE: Synthesis of the FA-CuS NPs is achieved using a one pot synthesis method adapted from a previously published protocol[21].

CAUTION: All synthesis should occur in a ventilated chemical fume hood.

1.1. Prior to synthesis, filter approximately 300 mL of deionized (DI) water though a 0.2 µm sterile filter.

1.2. Clean a 250 mL glass round bottom flask with a detergent solution and rinse with DI water. Add 0.0134 g of $CuCl_2$ into 100 mL of DI water to create a 1 mM solution.

1.3. Add 0.015 g of folic acid (FA) to the $CuCl_2$ solution and stir for ~5 min using a magnetic stir bar.

1.4. Add $Na_2S{\cdot}9H_2O$ (0.024 g in 100 µL DI water) over approximately 10 s to the reaction mixture utilizing a 200 µL pipette.

NOTE: Upon addition of the $Na_2S{\cdot}9H_2O$, the solution will change color from a light yellow to a dark brown.

1.5. Cap the reaction and place in an oil bath, set to 90 °C, and continue stirring with a magnetic stir bar. After approximately 15 min, or when the oil bath has reached 85–90 °C, allow the reaction to proceed for an additional hour. Your mixture should gradually turn to a dark green color.

NOTE: Make sure to vent the system while heating the reaction mixture to avoid pressure buildup.

1.6. Remove the reaction vessel from the oil bath and briefly cool at room temperature for approximately 10 to 15 min before transferring to an ice bath.

1.7. Once the reaction mixture has cooled below 20 °C, adjust the pH to 10 utilizing 1M NaOH to dissolve the remaining folic acid into solution.

1.8. Purify the FA-CuS reaction mixture using a 30 kDa centrifugation column. Add solution in 15 mL batches to the column and centrifuge at 3,082 x *g* for 15 min.

1.9. Once all of the reaction mixture has been concentrated, recombine the concentrated fractions and wash 4x with 15 mL of pH 10 NaOH in the 30 kDa centrifugation column.

1.10. Take 1/3 of the solution (~66 µL) and split into three glass vials. Dry in a vacuum oven overnight at 40 °C under a vacuum of ~27 mmHg.

1.11. Dissolve the other 2/3 of concentrated solution into 250 µL of PBS and store at 4 °C until further use.

1.12. Prior to utilizing the FA-CuS NPs, sonicate them for 30 min in a bath sonicator on a high setting.

2. NP characterization

2.1. Perform dynamic light scattering (DLS): Add 10 µL of concentrated FA-CuS NPS in PBS solution from step 1.1.14 to 2 mL of DI water. Prior to characterization by DLS, sonicate the particles for 30 min in a bath sonicator on a high setting and filter through a 0.2 µm sterile filter to remove residual dust.

2.2. Perform transmission electron microscopy (TEM)**:** Add 10 µL of concentrated FA-CuS NPS in PBS solution to a piece of wax paper. Invert a formvar coated copper grid on the top of the droplet and let sit for 2 min. Touch the edge of the formvar-coated grid to a piece of filter paper to remove excess liquid. Let air dry. Image the copper grid utilizing an electron microscope at an accelerating voltage of 80 kV.

NOTE: Typical results from FA-CuS NPS characterization are presented in **Figure 1**.

3. Cell culture

3.1. This protocol utilizes SKOV-3 cells. Unless otherwise noted, culture SKOV-3 cells in McCoy's 5A medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, and maintain at 37 °C in a humidified 5% CO_2 incubator.

4. Fluorescent tagging of FA-CuS NPS for microscopy

4.1. Add Texas-Red-X succinimidyl ester (0.2 mg dissolved in DMSO at a concentration of 10 mg/mL) to a solution containing 2 mg of FA-CuS NPs in 1 mL of 0.1 M $NaHCO_3$ (pH ~9) buffer.

4.2. Stir the reaction mixture using a magnetic stir bar for 1 h, away from light at room temperature.

4.3. Concentrate the reaction mixture in a 4 mL 30 kDa MWCO centrifugation column by spinning at 3,082 x *g* for 10 min.

4.4. Wash the concentrated solution 3x with 4 mL of 0.1 M $NaHCO_3$ buffer (pH ~9) in a centrifugation column. Subsequently, wash the concentrated solution with 4 mL of DI water 3x or until only a trace amount of fluorescence remains visible in the flowthrough by UV-VIS.

5. FA-CuS NPS uptake by ovarian cancer cells

5.1. Prior to incubation with FA-CuS NPs, incubate SKOV-3 cells in a T75 flask with 8−15 mL of folic-acid-free RPMI-1640 media with 10% FBS and 1% penicillin/streptomycin for at least 24 h.

5.2. Seed cells in 0.5 mL of folic-acid-free RPMI-1640 complete growth media at a density of 0.05 x 10^6 cells/mL into a 24 well plate.

5.3. The following day, incubate cells with 400 µg/mL FA-CuS NPS in 0.5 mL of folic-acid-free RPMI-1640 complete growth media for 2 h.

5.4. Following this incubation, trypsinize the cells with 0.5 mL of 0.25% trypsin with EDTA. Add at least 1 mL of folic-acid-free RPMI-1640 complete growth media to neutralize the trypsin, and centrifuge the cells at 123 x *g* for 6 min.

5.5. Remove the supernatant, resuspend the cells in 2 mL of PBS, and centrifuge at 123 x *g* for 6 min. Perform this wash step 2x to remove any unbound NPs.

5.6. Resuspend the cells in 1−2 mL of PBS with 2% Tween solution.

5.7. Count the cells using a hemocytometer and trypan blue. Further dilute cells if cell counts are too high. Dilute cells in PBS with 2% Tween to the chosen concentration for detection.

5.8. The cells are now ready to be analyzed by the PAFC system.

6. Fluorescence microscopy of FA-CuS NPS uptake

6.1. Repeat steps listed in step 5.1 and proceed with protocol below for microscopy.

6.2. Seed cells at a density of 0.05 x 10^6 cells/mL in 0.05 mL of folic-acid-free RPMI-1640 complete growth media on glass coverslips in a 24 well plate.

6.3. The following day, incubate the cells with fluorescently tagged FA-CuS NPs in triplicate, at concentrations of 100 µg/mL, 200 µg/mL, 300 µg/mL, and 400 µg/mL in 0.5 mL of folic-acid-free RPMI-1640 complete growth media.

6.4. Incubate the cells with the NPs for 2 h in the 37 °C incubator

6.5. Following this incubation period, wash the cells 3x with PBS.

NOTE: For all wash steps, carefully add the solution on the side of the well plate to not disturb the cells. After addition, carefully tilt the plate and withdraw the solution from the side of the well.

6.6. Incubate the cells with 0.5 mL of 3.7% paraformaldehyde (PFA) in PBS for 15 min and transfer the glass coverslips to a new 24 well plate.

CAUTION: PFA is a known carcinogen. Do all fixation in a ventilated chemical fume hood and wear appropriate personal protective equipment.

6.7. Incubate the cells in a solution of 3.7% PFA with 0.1% Triton-X in PBS for 5 min.

6.8. Wash the cells with 0.5 mL of PBS 3x for 5 min each and transfer the coverslips to a new plate.

6.9. Incubate the cells with 0.5 mL of a PBS solution containing DAPI (20 µL/mL of a 0.5 mg/mL stock solution is used for staining) for 5 min, away from light.

6.10. Wash the cells with PBS 3x.

6.11. Following the final PBS wash, mount the coverslips on slides with mounting medium.

6.12. The cells are now ready to be imaged by fluorescence microscopy. **Figure 2** shows an example of typical cell characterization by fluorescence microscopy.

7. Flow system architecture

7.1. Flow chamber construction

NOTE: A SolidWorks file of a 3D printed flow tank can be found in the **Supplementary Materials**.

7.1.1. Using the provided SolidWorks file, 3D print the flow tank using ABS thermoplastic or PLA plastic. Dimensions are provided below if SolidWorks is unavailable. **Figure 3A** shows a representation of this model. The body of the flow tank is 2.5 cm x 1.5 cm x 7.5 cm. The far ends of the flow tank include holes approximately 5 mm in diameter to allow for the entry of tubing containing the capillary tube.

NOTE: The flow tank has a 1 cm hole, perpendicular to the orientation of the capillary tube, for placement of the ultrasound transducer. A cylindrical extrusion with the same inner diameter as the hole extends 6 mm into the tank. For real-time imaging, the flow tank has a 1 mm x 3 mm slot directly below the capillary tube.

7.1.2. After printing the 3D flow tank, clean and assemble the system for use.

7.1.3. Place glass coverslips over the 1 mm x 3 mm slot and the 1 cm hole in the flow system.

7.1.4. Carefully seal with silicone to prevent leakage.

7.1.5. Fit the capillary tube into the silicone cured tubes. Insert the tubes into the flow chamber though the side of the flow tank such that the glass capillary tube is directly above and in front of the 3 mm slot and the 1 cm hole.

7.1.6. Seal the tubing using silicone to prevent leakage.

7.2. Photoacoustic flow system setup

NOTE: **Figure 3B** and **Figure 3C** show an example of the flow system architecture.

7.2.1. Connect the transducer to an ultrasound pulser/receiver. Amplify the signal with a 59 dB gain.

7.2.2. Connect the output of the filter to a multipurpose reconfigurable oscilloscope equipped with a built-in field programmable gate array.

7.2.3. Connect one of the tubes coming from the flow chamber to a T-junction, connected to two syringe pumps at each branch.

7.2.4. Fill one of the syringe pumps with air and the other pump with the sample to be analyzed. Set the pump containing air to a flow rate of 40 µL/min and the pump containing the sample to a flow rate of 20 µL/min. The resulting two-phase flow will produce sample volumes of 1 µL. At this flow rate, the system will test approximately 6.4 samples per minute.

NOTE: To maintain a consistent distribution of cells, lightly vortex each sample immediately before being tested. In addition, rotate the syringe every few minutes in order to prevent the cells from settling in the solution.

7.2.5. Connect the remaining tube exiting the flow system to a container with 10% bleach, to dispose of cells after they exit the flow system.

NOTE: Before utilizing the flow system, check for leaks, as these can affect the flow. Cells must be contained within a closed system to maintain biological safety during the procedure.

7.2.6. The design of the 3D printed tank allows for consistent and repeatable alignment between the transducer and laser light with minimal calibration. When placed correctly within the custom tank, the quartz capillary tube ensures that the transducer and laser are directly aligned.

7.2.7. Place the section of the quartz capillary tube in direct alignment with the transducer, in the field of view of the microscope, allowing for careful placement of the optical fiber above the sample such that it illuminates the entire width of the tube.

7.2.8. Irradiate the sample using an optical fiber channeling a diode-pumped solid state laser operating at a wavelength of 1,053 nm. The laser light incident on the sample and the transducer used to measure the photoacoustic effect are both unfocused.

7.2.9. The energy of the laser incident on the sample is approximately 8 mJ and the 10 Hz laser rate is sufficient to illuminate each sample multiple times as it passes through the system.

7.2.10. Place the flow system on top of an inverted microscope and ensure both the laser pulse and the path of the sample are visible as the sample passes though the flow system. Record flow using a microscope-mounted camera.

7.2.11. Record the ultrasound acquisitions utilizing data acquisition software (see **Table of Materials**). Trigger ultrasound and pulsed laser using the FPGA. Utilize PBS with 2% Tween, and FA-CuS NPs at a concentration of 100 µg/mL in PBS 2% Tween, as negative and positive controls, respectively.

7.2.12. Utilizing a microscope-mounted camera, record both the firing of the laser and the passage of samples though the flow system. These recordings will be utilized to correlate the acoustic signal recorded by the transducer with the firing of the laser. As the samples pass in front of the firing of the laser, the signal can then be correlated to the resulting photoacoustic signal for analysis. At a sampling rate of 10 Hz, the laser will illuminate each plug several times.

8. Post processing

8.1. For each signal acquisition, s(t), calculate the Hilbert transform, H[s(t)], in order to create an analytic signal.

8.2. Create a complex envelope, $s_e(t)$, by calculating the magnitude of the analytic signal, such that

$$s_e(t) = \sqrt{(s^2(t) + H^2[s(t)])}$$

and integrate the envelope to measure the total signal resulting from each acquisition. Compare the signals from each test group (i.e., PBS, tagged cells, FA-CuS NPs, cells alone) utilizing a t-test in R statistical software. Raw photoacoustic signals and their Hilbert transforms are presented in **Figure 4**.

8.3. For image reconstruction, normalize the complex envelope based on the maximum peak across the whole run. If comparing a series of runs, normalize the complex envelope using the maximum peak across the entire series. Following normalization, convert each acquisition into a

series of pixel values. Represent each series of pixel values as a column in the image reconstruction. Representative reconstructions of PBS and the FA-CuS NPs signals are shown in **Figure 5**, where both images were normalized using the maximum peak across both runs**.**

REPRESENTATIVE RESULTS:

Figure 1A shows a typical TEM image of the synthesized nanoparticles. The average size of the typical nanoparticle is approximately 8.6 nm ± 2.5 nm. Nanoparticle measuring was performed in ImageJ. Threshold and watershed functions were applied to separate the particles for measurement. The horizontal and vertical diameters of each particle were measured perpendicular to each other and further averaged. For DLS, a representative measurement is shown in **Figure 1B**. The average hydrodynamic diameter for these particles is 73.6 nm. Copper sulfide nanoparticles have a characteristic absorbance curve which extends into the NIR, as shown in **Figure 1C**. There is a slight artifact around 850 nm that was caused by the switching of lasers by the spectrophotometer

Fluorescence microscopy images of cells incubated with fluorescently tagged nanoparticles can be seen in **Figure 2**. Nanoparticle uptake can be visualized by the presence of fluorescence across the cell. Cells not incubated with nanoparticles show no fluorescence signal. The presence of this fluorescence signal indicates the successful uptake of the particles and their ability to be detected in the flow system.

Figure 3 shows the general setup of the photoacoustic flow system. **Figure 3A** shows a detailed model of the 3D flow chamber. This chamber can be printed utilizing the .stl file provided with this protocol. **Figure 3B** shows an overview of the flow tank setup. **Figure 3C** shows a general setup of the flow tank and data acquisition system.

Typical data acquisition signals are shown in **Figure 4**. The raw data indicates the differences in signal between the nanoparticle tagged cells, PBS, and FA-CuS NPs. An acquisition is the resulting photoacoustic signal generated from a single laser pulse. Due to the rapid firing rate of the laser, each sample analyzed generates multiple acquisitions. An envelope for each individual acquisition was generated using the Hilbert transform. This envelope was integrated to measure the total amount of signal generated from the laser pulse. In a previous study, these data were analyzed using R statistical software, where the number of acquisitions analyzed for the t-test were 203, 150, 160, and 131, for cells with NPs, cells alone, PBS, and NPs alone, respectively[22]. The data were normalized by log transformation and compared utilizing a Welch's t-test in R. The signals resulting from the FA-CuS NPs alone at a concentration of 100 µg/mL showed a much higher signal than the negative control. The difference in the signals between the negative control and the tagged ovarian CTCs were more subtle than the positive control but could be detected through the analysis of their means by a t-test[22].

Utilizing custom LabView and MATLAB software, image reconstructions were made of the positive and negative controls in real-time and post-acquisition, respectively. In order to generate the photoacoustic reconstructions, an envelope of each acquisition was calculated using the Hilbert transform. Individual envelopes were subsequently converted into pixel values

and displayed as independent columns. Clear differences in photoacoustic signal occur between the FA-CuS NPs at a concentration of 100 µg/mL and the PBS sample (**Figure 5**). Controls for the system are important to run to ensure that the system is adequately producing photoacoustic signal that can be detected by the transducer.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative NP characterization. (**A**) TEM image of synthesized FA-CuS NPs. Scale bar = 50 nm. (**B**) Representative DLS intensity distribution of synthesized FA-CuS NPs. (**C**) Representative FA-CuS NPs absorbance curve.

Figure 2: Representative fluorescence microscopy images of SKOV-3 cells. The cells were incubated with and without 400 µg/mL fluorescently-tagged NPs. Scale bar = 50 µm.

Figure 3: Representative images of photoacoustic flow cytometry system and flow chamber. (**A**) Detailed view of the 3D printed flow chamber. (**B**) Diagram of PAFC system. (**C**) Flow system architecture: SP = syringe pump; DAQ/FPGA = data acquisition/field programmable gate array; Ob = objective lens; OF = optical fiber; FC = fiber coupler; UT = ultrasound transducer; FT = flow tank. This figure is adapted from Lusk et al.[22].

Figure 4: Representative raw data signal and Hilbert transforms of samples tested in the flow system. (**A**) Representative raw data signal from PBS and cells incubated with NPs and the (**D**) Hilbert transform of the data. (**B**) Representative raw data signal from the cells alone and the cells incubated with FA-CuS NPs and (**E**) the Hilbert transform of the data. (**C**) Representative raw data signal from PBS and 100 µg/mL FA-CuS NPs and (**F**) the Hilbert transform of the data.

Figure 5: Representative photoacoustic image reconstructions of the photoacoustic data. (**A**) Image reconstruction of 100 µg/mL FA-CuS NPs and (**B**) PBS tested within the flow system.

DISCUSSION:

This protocol is a straightforward method for the detection of ovarian CTCs utilizing PAFC and a targeted CuS contrast agent. Many methods have been explored for the detection of ovarian CTCs, including microfluidic devices, RT-PCR, and fluorescence flow cytometry[23-25]. These range in complexity, cost, and accuracy, limiting their effectiveness in clinical settings. PAFC introduces several advantages over these traditional methods for the detection of CTCs, including the ability to detect CTCs within patient samples, and its ease of translation to in vivo applications. PAFC has also been shown to accurately detect CTCs in vitro and in vivo when combined with targeted contrast agents[26,27].

In this work, FA-CuS NP contrast agents were evaluated for their ability to improve the accuracy of CTC detection at physiologically relevant concentrations. Studies were performed using isolated SKOV-3 cells resuspended in PBS. Analysis by fluorescence microscopy indicated the successful uptake of these particles. The results detected SKOV-3 cells down to a concentration of 1 cell/µL[22]. Crucial steps in this protocol involve the nanoparticle synthesis and the alignment of the photoacoustic flow setup. During nanoparticle synthesis, it is important to make sure that

the solution has turned a dark green color before removing the mixture from the oil bath. For the flow system, running a negative and positive control though the system prior to sample testing is necessary to ensure that the system is producing adequate photoacoustic signal for subsequent detection of labeled cells. While running the flow system, it is important to ensure that the incident light from the fiber optic is completely illuminating the capillary tube, and that the transducer is snug against the side of the flow chamber. Finally, rigorous testing of the system for leaks is necessary to ensure the biological safety of the system and for consistent flow through the chamber.

For the current PAFC system, preliminary studies confirmed photoacoustic detection using the transducer and amplification system. The majority of these signals were comprised of lower frequency signals (<20 MHz). Further studies are needed to confirm whether this is due to the actual frequency of the generated photoacoustic signals or the 35 MHz bandwidth of the amplifier. Future studies will investigate the frequency components of the detected signals in order to optimize the central frequency of the transducer as well as the bandwidth of the amplification system. The current system is specifically suited for ex vivo detection of CTCs. However, this method identifies the potential for future application of FA-CuS NPs in vivo.

Future studies aim to detect ovarian cancer cells within mixed cultures, human clinical samples, and in vivo models[28-30]. Furthermore, future studies will examine the specificity of these nanoparticles versus non-targeted controls. Future validation of this method will include testing this tool with human clinical samples, implementation of high throughput testing and analysis, and translation into clinical settings. This photoacoustic technique shows potential for future translation to a wide variety of clinical applications and diseases across a range of contrast agents and analytes. Photoacoustic detection of analytes utilizing PAFC has the potential to make the point-of-care detection of CTCs and other pathogens more rapid and inexpensive.

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DISCLOSURES:

The authors have nothing to disclose.

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circulating tumour cells. *Nature Nanotechnology.* **4** (12), 855 (2009).

Figure 1

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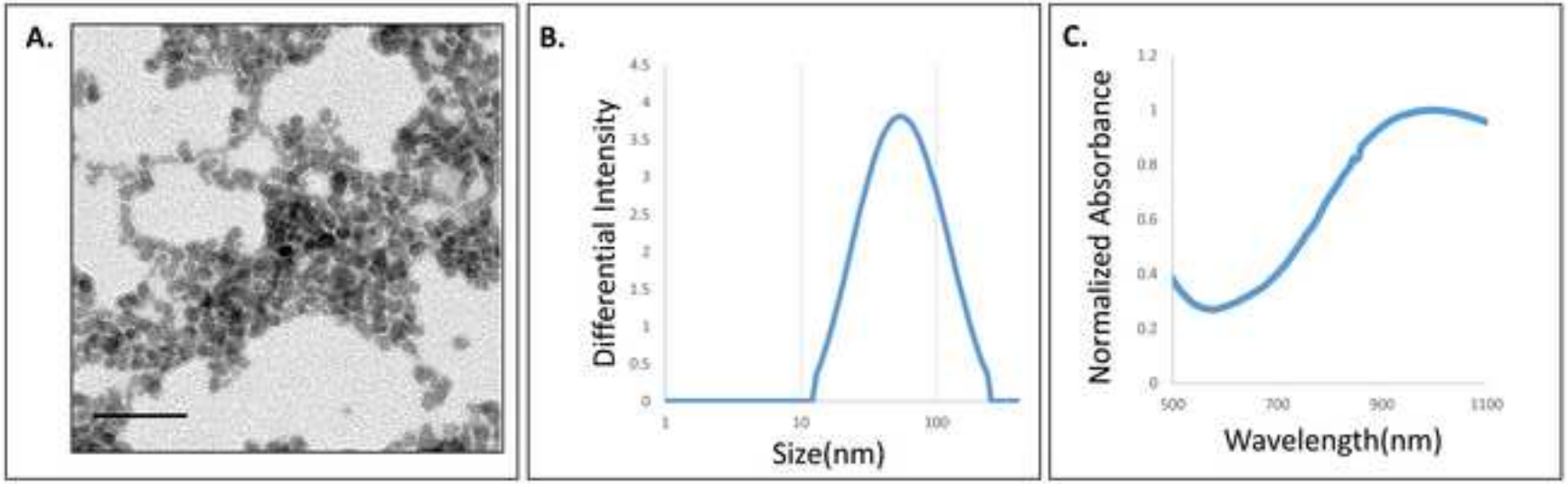

Figure 2

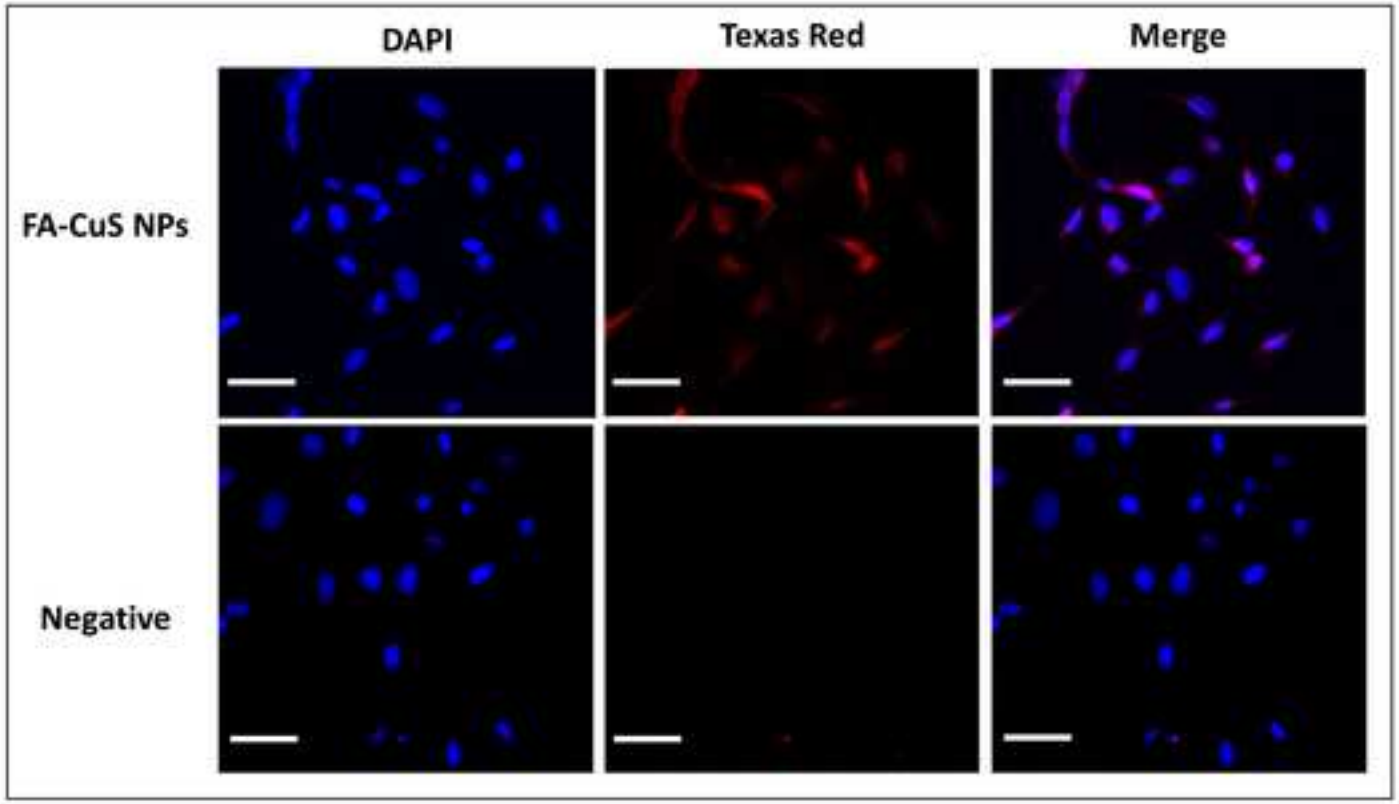

Figure 3

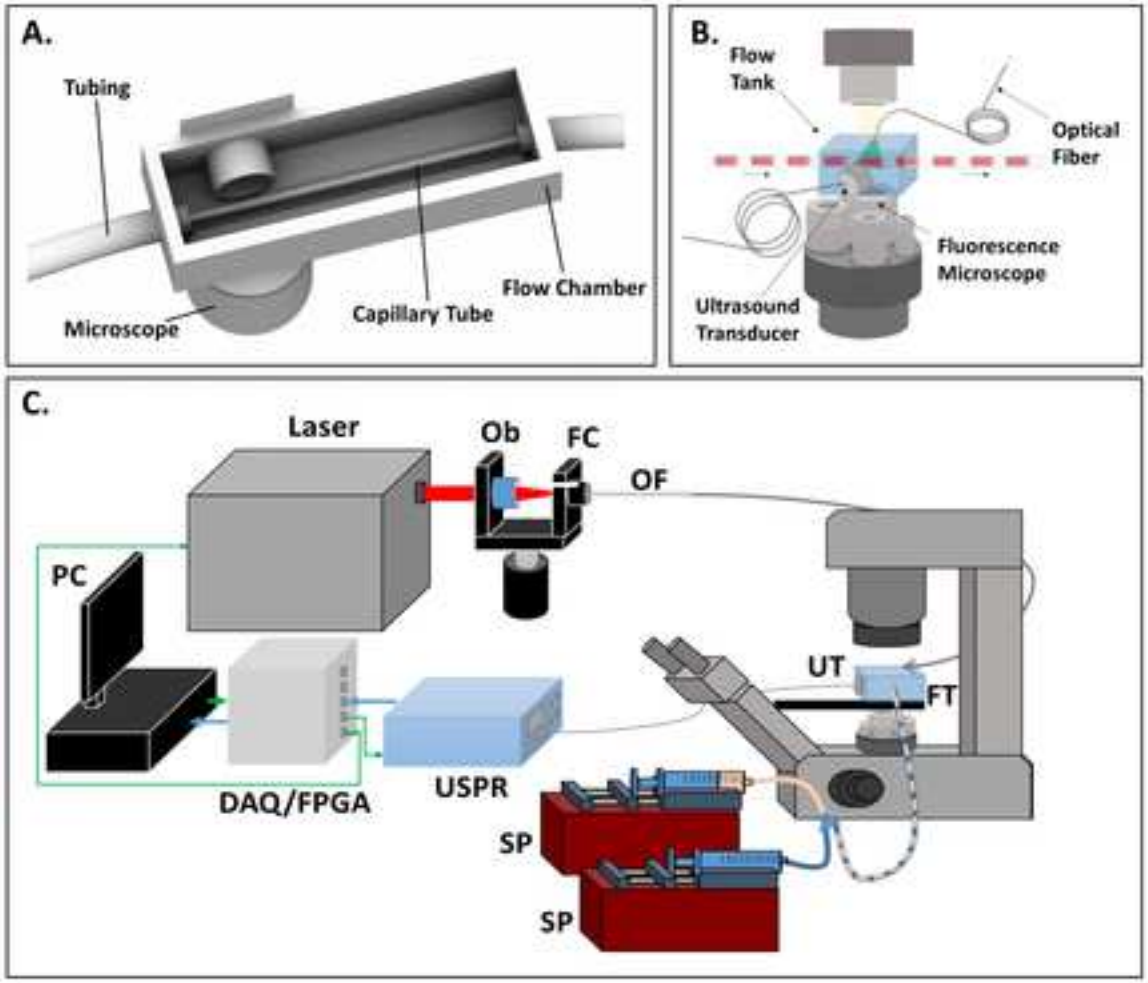

Figure 4

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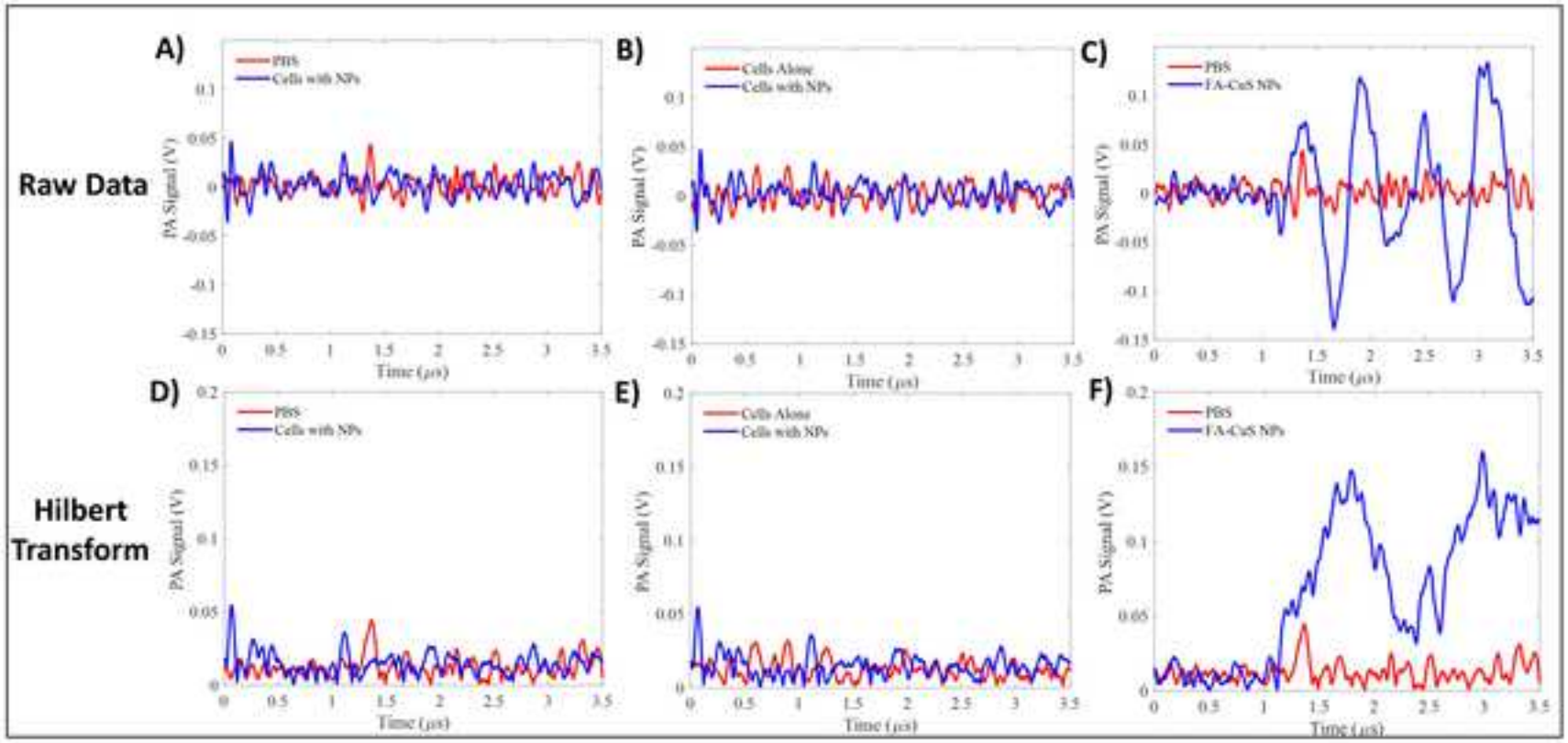

Figure 5

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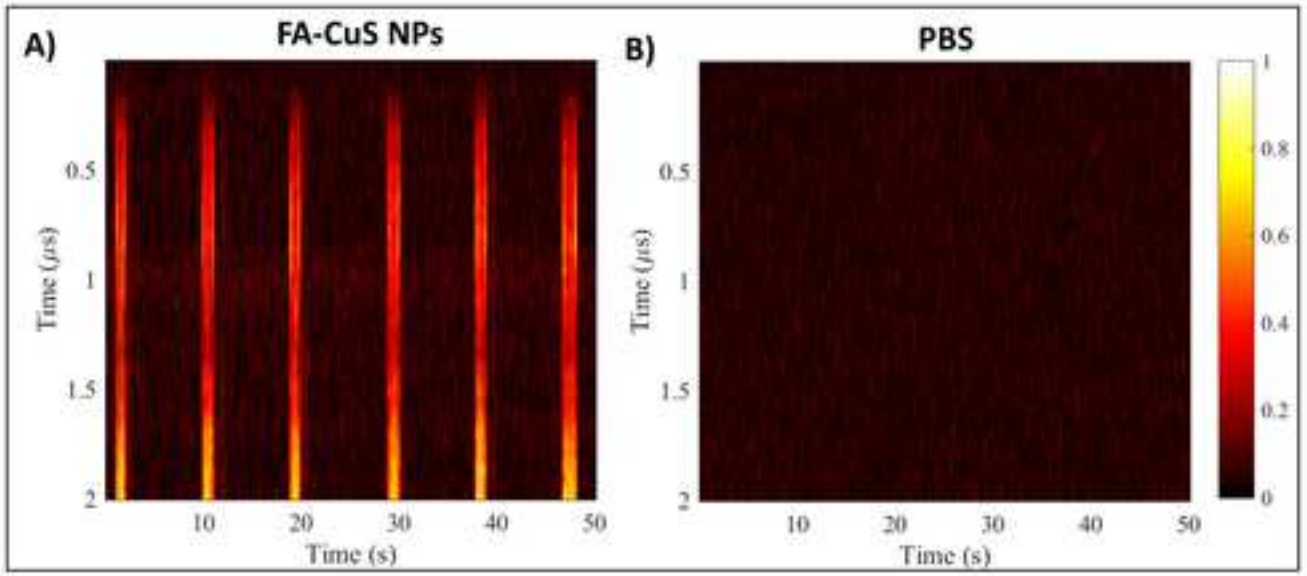

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.025% Trypsin With EDTA	Corning	25-053-Cl	
0.2 µm 1000 mL Vacuum Filtration Unit	VWR	10040-440	For filtering larger volumes of DI water.
0.2 µm sterile syringe filter	VWR	28145-477	
3D Printed Tank		Custom-made	
Acquisition Card	National Instruments	PXIe-5170R	250 MS/s, 8-Channel, 14-bit
Alconox	Sigma-Aldrich	242985-1.8KG	Detergent used for cleaning glassware.
Amicon Ultra-15 Centrifugal Filters	Millipore	UFC903024	
Amicon Ultra-4 Centrifugal Filters	Millipore	UFC803024	
Bright-Line Hematocytometer	Hausser Scientific	1492	
Copper(II) Chloride	ACROS ORGANICS	206532500	
Coupling Objective	Thorlabs	LMH-10x-532	To couple pulsed light to optical fiber.
Coupling Stage	Newport	F-91-C1-T	Stage for coupling pulsed light to objective. Holds FP-1A and LMH-10x-532
CPX Series Digital Ultrasonic Cleaning Bath	Fisherbrand	Model CPX3800	
Data Acquisition software	National Instruments	NI LabVIEW 2017 (32-bit)	LabVIEW used to synchronize laser pulses with data acquisition.
Data Processing Software	Mathworks	Matlab R2016a	Reconstructions and graphs produced using Matlab software.

FBS	Sigma-Aldrich	F2442-500ML	
Fiber Chuck	Newport	FPH-DJ	Used to hold the bare fiber.
Fiber Coupler	Newport	FP-1A	3-Axis stage for positioning fiber chuck and optical fiber at the focus of the objective.
Folic Acid	Sigma-Aldrich	F7876-10G	
Formvar Coated TEM Grids	Electron Microscopy Sciences	FCF300-CU-SB	
Masterflex Tubing	Cole Parmer	EW-96420-14	
McCoy's 5A Medium	ATCC	30-2007	
Norm-Ject 10 mL Syringes	HENKE SASS WOLF	4100-X00V0	
Optical Fiber	Thorlabs	FG550LEC	Used to expose sample to pulsed light.
PBS	Alfa Aesar	J62036	
Penicillin Streptomycin	GIBCO	15140-122	
Pulsed Laser	RPMC Lasers Inc	Quantus-Q1D-1053	Pulsed laser source with specifications 1053 nm, 8 ns pulse, 10 Hz maximum.
Pulser/Receiver	Olympus	5077PR	Receives, filters, and amplifies photoacoustic signals. Operated with 59 dB Gain.
Quartz Capillary Tube	Sutter Instrument	QF150-75-10	
RPMI Midum 1640 (1X) Folic Acid Free	Gibco	27016-021	

Silicone	Momentive Performance Materials, Inc.	GE284	
SKOV-3 Cells	ATCC	HTB-77	
Sodium Bicarbonate	Sigma-Aldrich	S5761	
Sodium Carbonate	Sigma-Aldrich	S7795-500G	
Sodium Hydroxide Beads	BDH	BDH9292-500G	
Sodium Sulfide Nonahydrate	Sigma-Aldrich	431648-50G	
Syringe Pumps	New Era Pump Systems Inc	DUAL-1000	
Texas Red-X-Succinimydl ester	Invitrogen	1949071	
Transducer	Olynmpus	V214-BB-RM	Ultrasound detector with central frequency of 50 MHz and -6 dB fractional bandwidth of 82%.
Trypan Blue Solution .4%	Amresco	K940-100ML	
Tween 20	Sigma-Aldrich	P7949-100ML	
Ultrasound Gel	Parker Laboratories Inc.	Aquasonic 100	Ultrasound gel for transducer coupling

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Author(s): Joel F. Lusk, Christopher Miranda, and Barbara S. Smith

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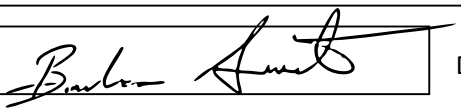
Signature:	 Date: 16 May 2019

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We thank the Reviewers for their feedback. We have addressed each one of their questions in the text below. Our responses and updates to the manuscript have been made to each of the reviewers in a corresponding color, to make it easier to refer to: Reviewer 1: blue, Reviewer 2: green, and Reviewer 3: orange, and the Editor: red. For ease of review, we have broken up each of the comments into smaller segments and have addressed them individually, included below.

Response to Reviewer 1:

Reviewers' comments:

Manuscript Summary:

Joel F. Lusk, Christopher Miranda, and Barbara S. Smith have submitted a manuscript to JoVE entitled, "Ovarian Cancer Detection Using Photoacoustic Flow Cytometry" describing the methods to synthesize folic acid conjugated copper sulfide nanoparticles, their characterization, labeling of cells with the nanoparticles, and finally the setup of a photoacoustic flow cytometry system that can detect down to "individual cells." Overall the ideas behind this manuscript are extremely interesting and the methodologies could be of use to many people. While better after resubmission, the manuscript protocol is still severely lacking in details and specificity and, as is, would be difficult to follow the protocol to produce the results. The major concerns now also include a lack of clarity on the data acquisition timing/registration between visual microscope images and PA signals and data analysis methods.

> We thank Reviewer 1 for the feedback. We have addressed each of your questions, as included below. Our responses have been made in blue, so that it is easy to reference, and corresponding updates to the manuscript are also in blue. We have broken up each of the comments into smaller segments and have addressed them individually.

Please see more specific comments as follows:

Introduction

Line 70. Please add a reference(s) concerning the luminal expression of folate receptors vs any exposure in the vasculature system.

We have included a citation in the following portion of the manuscript to reference the luminal expression of folate receptors:

> *"Folate receptor targeting is an ideal strategy for the identification of ovarian CTCs in blood due to the fact that endogenous cells, which have higher expression of folic acid receptors, are generally luminal and have limited exposure to the blood stream.[1]"*

1. Cheung, Anthony, et al. "Targeting folate receptor alpha for cancer treatment." *Oncotarget* 7.32 (2016): 52553.

Line 82. "These results show detection of ovarian cancer cells down to concentrations of 1 cell/µl" The authors do not demonstrate this claim in the manuscript with the results as currently represented. Please adjust or reference to a paper where this detection limit was accomplished.

We have included a reference to our previous manuscript where we show the detection of ovarian cancer cells down to 1 cell/ µL, as indicated in the following passage:

> *"These results show detection of ovarian cancer cells down to concentrations of 1 cell/µL, and fluorescence microscopy confirms successful uptake of these particles by SKOV-3 ovarian cancer cell.*[1]*"*

1. Lusk, Joel F., et al. "Photoacoustic Flow System for the Detection of Ovarian Circulating Tumor Cells Utilizing Copper Sulfide Nanoparticles." *ACS Biomaterials Science & Engineering* 5.3 (2019): 1553-1560.

Protocol:

1.1.2 ◊ What total volume of water should be filtered for this protocol?

We have updated this portion of the protocol to reflect the approximate total amount of water needed for the entire protocol.

> *"1.1.2: Prior to synthesis, filter approximately 300 mL of DI water through a 0.2 µm sterile filter."*

1.1.3 ◊ Are any special cleaning steps required for the glassware prior to synthesis? (i.e. Aqua Regia? Etc?)

The manuscript has been updated to indicate that detergent and DI water were used to clean all glassware.

> *"1.1.3: Add $CuCl_2$ into 100 mL of DI water to create a 1 mM solution (0.0134 g) in a 250 mL round bottom flask. Prior to use, clean glassware with an detergent solution and rinse with DI water."*

1.1.4 ◊ Stir with what? A scoopula? A magnetic stir bar?

We have adjusted the manuscript to indicate that we utilized a magnetic stir bar to stir the solution.

> *"1.1.4: Add folic acid (FA) to the solution (0.015 g) and stir with the $CuCl_2$ for ~5 min using a magnetic stir bar."*

1.1.5 ◊ How slowly? Drop wise? A specific volume/time?

We have updated the manuscript to include the rate at which the sodium sulfide is added to the solution.

> *"1.1.5: Add $Na_2S{\cdot}9H_2O$ (0.024 g in 100 µL DI water) over approximately 10 sec to the reaction mixture utilizing a 200 µL pipette."*

1.1.6 ◊ This step would be better as a "Note"

We have updated the manuscript to include this section as a note:

> *"NOTE: Upon addition of the Na2S·9H2O, the solution will change color from a light yellow to a dark brown."*

1.1.7 ◊ This step is unclear. Is the oil cool when the reaction vessel is placed in it and then allowed to warm to 90 C? Is the mixture stirred during this?

The manuscript has been updated to clarify this section.

> *"1.1.6: Cap the reaction and place in a cool oil bath, set to 90°C and continue stirring with a magnetic stir bar."*

1.1.8 ◊ Briefly cool to what temperature and for how long?

The manuscript has been clarified to indicate the temperature at which the reaction vessel is cooled, and the duration of cooling, before transferring to an ice bath.

> *"1.1.7: Remove the reaction vessel from the oil bath and briefly cool at room temperature for approximately 10 to 15 min before transferring to an ice bath."*

1.1.9 ◊ What temperature should the reaction vessel reach before adjusting the pH?

We have updated the manuscript to indicate the temperature of the reaction vessel prior to adjusting the pH:

> *"1.1.8: Once the reaction mixture has been cooled below 20°C, adjust the pH to 10 using 1M NaOH to dissolve the remaining folic acid into solution."*

1.1.10 ◊ Would be clearer if it stated, "In 15 ml batches…" as opposed to one 15 ml step.

We have updated the manuscript to include the reviewer's recommendation.

> *"1.1.9: Purify the FA-CuS reaction mixture using a 30 kDa centrifugation column. Add solution in 15 mL batches to the column and centrifuge at 3082 g for 15 min."*

1.1.11 ◊ How much NaOH should be used for each wash? What is the final volume one might hope to reach of concentrated solution?

This portion of the manuscript has been updated to reflect the amount of NaOH used for each of the washes.

> *"1.1.10: Once all of the reaction mixture has been concentrated, recombine the concentrated fractions and wash 4 times with 15 mL pH 10 NaOH in the 30 kDa centrifugation column."*

1.1.12 ◊ Approximately what volume is 1/3 of the batch? What kind of tubes? What are the vacuum oven settings?

The manuscript has been updated to include the approximate amount of solution, type of tubes, and the vacuum oven settings.

> *"1.1.11 Take 1/3rd of the solution (~66 µL) and split into 3 glass vials. Dry in a vacuum oven overnight at 40°C ,under vacuum of ~27 Hg."*

1.1.13 ◊ Might say, "Dissolve the other 2/3rds of the concentrated solution"

We have updated the manuscript accordingly:

> *"1.1.12: Dissolve the other 2/3rd's of concentrated solution into 250 µL of PBS and store at 4°C until further use.*

1.1.14 ◊ Settings/type of the bath sonicator?

The make and model of the bath sonicator is a a Fisherbrand CPX Series Digital Ultrasonic Cleaning Bath (Model CPX3800) and has been included on our materials list. The high setting was used to sonicate the particles.

This has been indicated in the protocol in the follow areas:

> *"1.1.3: Prior to utilizing FA-CuS NPs, sonicate them for 30 min in a bath sonicator on a high setting."*

"DLS (Dynamic Light Scattering): Add 10 µL concentrated FA-CuS NPS in PBS solution from step 1.1.14 to 2 mL DI water. Prior to characterization by DLS (dynamic light-scattering), sonicate the particles for 30 min in a bath sonicator on a high setting and filter through a 0.2 µm sterile filter to remove residual dust.

2.1.1 ◊ What type of 0.2 um sterile filter?

For the filtration either a VWR 1000 mL 0.2 µM filter can be utilized for larger volumes of water (Cat: 10040-440), or a VWR sterile syringe filter (Cat:28145-477) can be used for smaller volumes. Both filters have been included in the materials list.

4.1.1 ◊ What volumes of DMSO and NaHCO3 solution should this reaction occur in?

The amounts of both DMSO and NaHCO3 solution utilized have been clarified in this portion of the manuscript:

"4.1.1: Add Texas-Red-X succinimidyl ester (0.2 mg dissolved in DMSO at a concentration of 10 mg/mL) to a solution containing 2 mg of FA-CuS NPs in 1 mL 0.1 M $NaHCO_3$ (pH ~ 9) buffer."

4.1.2 ◊ Stir with what?

The manuscript has been updated to indicate that the reaction was stirred using a magnetic stir bar.

"4.1.2: Stir the reaction mixture for 1 hr, away from light at room temperature using a magnetic stir bar."

4.1.4 ◊ What volumes of NaHCO3 buffer and water should be used for washing? Clarify if the trace amounts of fluorescence should be in the wash through portion.

The volumes of NaHCO3 buffer and DI water needed for washing have been updated in the manuscript.

"4.1.3: Concentrate the reaction mixture in a 4 mL 30 kDa MWCO centrifugation column by spinning at 3082 g for 10 min.

4.1.4: Wash the concentrated solution 3 times with 4 mL 0.1 M $NaHCO_3$ buffer (pH ~ 9) in a centrifugation column. Subsequently, wash the concentrated solution with 4 mL DI water 3 times or until only a trace amount of fluorescence remains visible in the flow through by UV-VIS."

5.1.1 ◊ This volume seems specific for a type of plate, please specify.

We thank the reviewer for this recommendation. The type of plate utilized in this study has been clarified in this section of the manuscript. The original document stated 0.5 mL of media, as this was the amount of media needed for a 24 well plate. However, in this protocol we primarily utilize T75 flasks with 8-15 mL media to grow cell culture prior to experiments. This has been clarified in the manuscript accordingly:

> *"5.1.1. Prior to incubation with FA-CuS NPs, incubate SKOV-3 cells in a T75 flask with 8-15 mL folic-acid-free RPMI-1640 media with 10% FBS and 1% pen/strep for at least 24 hours."*

5.1.3 ◊ What volume of media per well?

The manuscript has been updated to clarify the amount of media needed per well.

> *"5.1.2: Seed cells in 0.5 mL folic-acid-free RPMI-1640 complete growth media, at a density of 0.05 × 10^6 cells/mL into a 24 well plate."*

> *"5.1.3: Seed cells in 0.5 mL folic-acid-free RPMI-1640 complete growth media, at a density of 0.05 × 10^6 cells/mL into a 24 well plate."*

5.1.3 ◊ Volumes of trypsin and media per well?

We have updated the manuscript to describe the volumes of trypsin and media in each well.

> *"5.1.4: Following this incubation, trypsinize the cells with 0.5 mL of 0.25% trypsin with EDTA. Add at least 1 mL folic-acid-free RPMI-1640 complete growth media to neutralize the trypsin, and centrifuge the cells at 123 g for 6 min. "*

5.1.5 ◊ Volume of PBS?

The manuscript has been updated to include the amount of PBS used to wash the cells.

> *"5.1.5: Remove the supernatant, resuspend the cells in 2 mL of PBS, and centrifuge at 123 g for 6 min. Perform this wash step two times to remove any unbound NPs."*

5.1.6 ◊ Volume of PBS?

The manuscript has been updated to include the volumes of PBS in this step.

> *"5.1.6:* Resuspend the cells in 1-2 mL PBS with 2% TWEEN solution.

5.1.7: Count cells using a hematocytometer and trypan blue. Further dilute cells if cell counts are too high. Dilute cells in PBS with 2% TWEEN to the chosen concentration for detection."

6.1.2 ◊ What volume of media?

The volume of media has been clarified in this step of the manuscript:

> *"6.1.2: Seed cells at a density of 0.05 × 10^6 cells/mL in 0.05 mL folic-acid-free RPMI-1640 complete growth media on glass coverslips in a 24 well plate."*

6.1.3 ◊ Are these concentrations total for the volume in the well, or a specific volume of NP solutions at those concentrations is added?

These concentrations are total for the volume in the well. This has been clarified in the manuscript:

> *"6.1.3 The following day, incubate the cells with fluorescently tagged FA-CuS NPs in triplicate, at concentrations of 100 µg/mL, 200 µg/mL, 300 µg/mL, and 400 µg/mL in 0.5 mL folic-acid-free RPMI-1640 complete growth media.."*

6.1.6 ◊ Volume of PFA?

The volume of PFA used has been clarified in the manuscript.

> *"6.1.6: Incubate the cells with 0.5 mL of 3.7% paraformaldehyde (PFA) in PBS for 15 mins, transfer the glass coverslips to a new 24 well plate."*

6.1.7 ◊ Volume of PFA/Triton-X solution? Why is there addition PFA here?

We thank the reviewer for their question, the PFA is added with Triton-X to continue fixation of the cells during the permeabilization by Triton-X.

6.1.8 ◊ Volume of PBS?

We have updated this portion of the manuscript to include the amount of PBS used at this step.

> *"6.1.8: Wash the cells with 0.5 mL of PBS 3 times for 5 min each and transfer the coverslips to a new plate."*

6.1.9 ◊ Volume of PBS?

The volume of PBS for this step has been included in the manuscript:

> *"6.1.9: Incubate the cells with 0.5 mL of a PBS solution containing DAPI (20 µL/mL of a 0.5 mg/mL stock solution is used for staining) for 5 min, away from light."*

6.1.10 ◊ For this wash step and all others: Explain how the washing works, example : "Wash cells three times by adding 1 ml of PBS to each well, swirling gently, and aspirating carefully as to not remove cells from the cover slip." Or if you mean something different? Please describe.

The wash procedures have been updated accordingly as in indicated 6.1.5, this has been included below for ease of reference. Additionally, a note has been added to the text.

> *"6.1.5: Following this incubation period, wash the cells 3 times with PBS.*
>
> *NOTE: For all wash steps, carefully add solution on the side of the well plate as to not disturb the cells. After addition, carefully tilt the plate and withdraw the solution from the side of the well."*

7.2.2 ◊ Why did the authors use a 50 MHz transducer (82% fractional BW) to detect a 2 MHz signal?

The use of a 50 MHz transducer has been discussed below and included in the manuscript on page 11.

> *"For our current PAFC system, preliminary studies confirmed photoacoustic detection using the transducer and amplification system. The majority of these signals are comprised of lower frequency signals (<20 MHz). Further studies are needed to confirm whether this is due to the actual frequency of the generated photoacoustic signals or the 35 MHz bandwidth of the amplifier. Future studies will investigate the frequency components of the detected signals in order to optimize the central frequency of the transducer as well as the bandwidth of the amplification system."*

7.2.5 ◊ How does the cell solution remain suspended to prevent clumping for even cell distribution throughout the experiment? What concentration of native NPs is being used for the control?

An even distribution of cells was maintained in the experiment, as explained below and included in the manuscript.

> *7.2.5 Fill one of the syringe pumps with air and the other pump with the sample to be analyzed. Set the pump containing air to a flow rate of 40 µL/min and the pump containing the sample to a flow rate of 20 µL/min. The resulting two-phase flow will produce sample volumes of 1 µL. At this flow rate, the system will test approximately 6.4 samples per minute.*
>
> *NOTE: To maintain a consistent distribution of cells, lightly vortex each sample immediately before being tested. In addition, rotate the syringe every few minutes in order to prevent the cells from settling in the solution.*

The concentration of the NP(control) has been added to the manuscript in the following section:

> *"7.2.12: Record the ultrasound acquisitions utilizing LabView software. Trigger ultrasound and pulsed laser using the FPGA. Utilize PBS with 2% TWEEN, and FA-CuS*

NPs at a concentration of 100 µg/mL in PBS 2% TWEEN as negative and positive controls, respectively."

7.2.10 ◊ What is the fluence?

The fluence of the laser has been listed in the following section in the manuscript:

"7.2.10: The energy of the laser incident on the sample is ~8 mJ. Results indicate that the 10 Hz laser rate is sufficient to illuminate each sample multiple times as they pass through the system."

7.2.11 ◊ How do you correlated the microscope video to the US/PA signals? Is it just by chance that a 1 ul aliquot is passing through the PA acquisition or is the system triggered somehow?

The recorded video of the laser firing is correlated to the US/PA signals. As the sample passes across the path of the laser.

"These recordings will be utilized to correlate the acoustic signal recorded by the transducer with the firing of the laser. As the samples pass in front of the firing of the laser, the signal can then be correlated to the resulting photoacoustic signal for analysis."

7.2.14 ◊ This is a repeated Note from 7.7.2.10. Please remove.

This section has been removed from the manuscript.

8.1.1 ◊ How many acquisitions are you comparing with the t-test? Is each acquisition the same length of time?

The amount of acquisitions analyzed for the t-test is listed in our original report[1]. The duration of each acquisition is the same length of time. The amount of acquisitions analyzed for the t-test were 203, 150, 160, and 131, for cells with NPs, cells alone, PBS, and NPs alone, respectively. We have added additional clarification in our manuscript, as well as provided a reference to the previous paper within the Data Acquisition section.

"In our previous study, this data was analyzed using R statistical software, where the amount of acquisitions analyzed for the t-test were 203, 150, 160, and 131, for cells with NPs, cells alone, PBS, and NPs alone, respectively.[1]"

1. Lusk, Joel F., et al. "Photoacoustic Flow System for the Detection of Ovarian Circulating Tumor Cells Utilizing Copper Sulfide Nanoparticles." *ACS Biomaterials Science & Engineering* 5.3 (2019): 1553-1560.

8.1.2 ◊ Normalize the complex envelope to what? The complex envelope of a single acquisition? This method is confusing. Please clarify.

The complex envelope is normalized based on the largest signal detected in either a whole run or a series of runs being compared. The manuscript has been updated to read:

> *"8.1.3: For image reconstruction, normalize the complex envelope based on the maximum peak across the whole run. If comparing a series of runs, normalize the complex envelope using the maximum peak across the entire series. Following normalization, convert each acquisition into a series of pixel values. Represent each series of pixel values as a column in the image reconstruction. Representative reconstructions of PBS and the FA-CuS NPs signals are shown in fig 5, where both images were normalized using the maximum peak across both runs. "*

Representative Results

*** Fluorescence Microscopy: A control of cells incubated with nontargeted NPs is missing from these images.

This protocol focused on the photoacoustic detection of cells in a flow system utilizing the cellular uptake of folic acid capped nanoparticles at physiologically relevant concentrations. The selectivity of these folic acid capped nanoparticles has been demonstrated in previous studies.[1] The current work combines photoacoustic targeting and ovarian cancer cellular uptake to identify circulating tumor cells at reduced concentrations. Future studies will examine the specificity of these particles over nontargeted copper sulfide particles. This is clarified in the discussion section of the manuscript:

> *"Future studies will examine the specificity of these nanoparticles versus non-targeted controls."*

1. Zhou, Min, et al. "Theranostic CuS nanoparticles targeting folate receptors for PET image-guided photothermal therapy." *Journal of Materials Chemistry B* 3.46 (2015): 8939-8948.

Flow System Architecture:

*** How is the optical fiber attached/aligned with the flow chamber/capillary tube? (7.2.8?)

The optical fiber alignment is explained in the following passage:

> *"7.2.8: Place the section of the quartz capillary tube in direct alignment with the transducer, in the field of view of the microscope. This allows for careful placement of the optical fiber above the sample, such that it illuminates the entire width of the tube."*

Data Acquisition:

*** How many acquisitions were in a group for the t-test? Analysis of what means by a t-test? Overall, the methods of data analysis are confusing and should be clarified further.

Relevant updates to the manuscript have been included in the data acquisition subsection of the representative results:

> *"In our previous study, this data was analyzed using R statistical software, the amount of acquisitions analyzed for the t-test were; 203, 150, 160, and 131, for cells with NPs, cells alone, PBS, and NPs alone, respectively.[1]"*
>
> 1. Lusk, Joel F., et al. "Photoacoustic Flow System for the Detection of Ovarian Circulating Tumor Cells Utilizing Copper Sulfide Nanoparticles." *ACS Biomaterials Science & Engineering* 5.3 (2019): 1553-1560.

Image Reconstruction:

What the authors are referring to as an "acquisition" is unclear. Is this a single US signal from a single laser pulse, the total signal from a 1 ul passage of sample through the capillary tube? Or?

An acquisition is the resulting photoacoustic signal generated from a single laser pulse. Because the laser can illuminate a sample multiple times, several acquisitions from each sample were acquired. This has been further clarified in the data acquisition subsection of the representative results:

> *"An acquisition is the resulting photoacoustic signal generated from a single laser pulse. Due to the rapid firing rate of the laser, each sample analyzed generates multiple acquisitions."*

Figures and Figure Legend

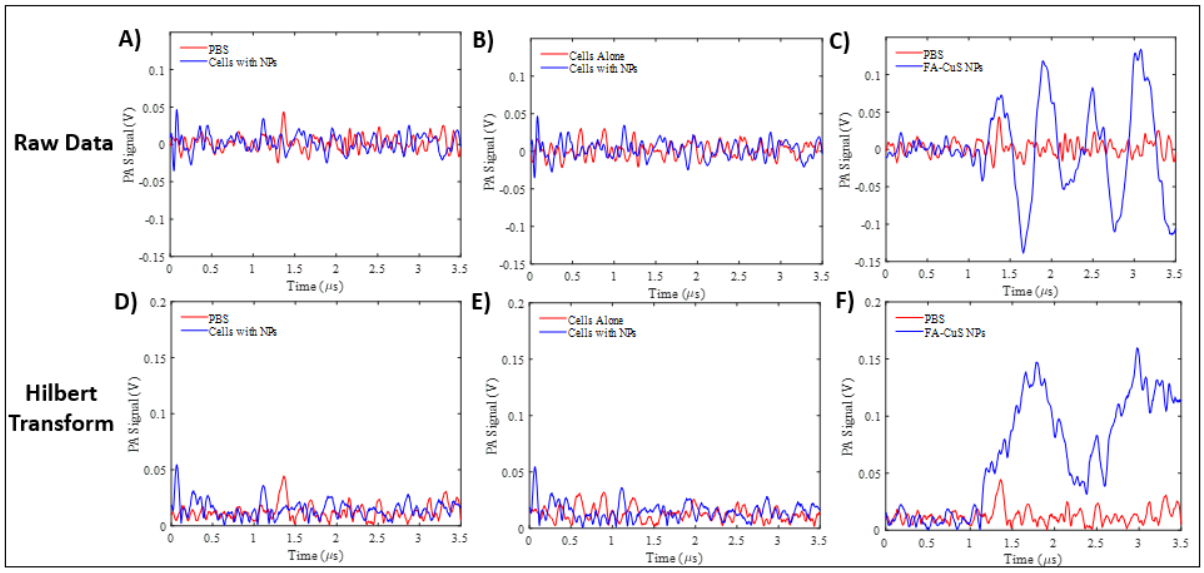
*** Figure 2 should also contain cell incubated with non-targeted CuS nanoparticles as a negative control.

We thank the reviewer for their feedback. This question has been addressed in the manuscript in the section below:

> *"Future studies will examine the specificity of these nanoparticles versus non-targeted controls."*

*** Figure 4: To better help the reader understand the signal and differentiate from noise, please plot the graphs to include a small amount of signal both before and after laser trigger.

The graphs have been updated with extended time points to better clarify signal differences.



In addition to the raw spectra presented here, please also include box plots showing what was compared in the t-tests. Such as, if there are 200 acquisitions for each condition (pbs, cells, cells+NP) please show the distributions of PA signal for each and the stdevs. This will help the reader visualize the "subtle" differences between the raw signal graphs.

A reference to our previous manuscript has been included in the representative results section. The referenced manuscript contains box plots of the t-test and also highlights the distributions of the signals and standard deviations.

> *"In our previous study, this data was analyzed using R statistical software, the amount of acquisitions analyzed for the t-test were; 203, 150, 160, and 131, for cells with NPs, cells alone, PBS, and NPs alone, respectively.[22] The data was normalized by log transformation, and compared utilizing a Welch's t-test in R."*

*** Figure 5. Why are the acquisitions routinely brighter at the end of the acquisition and low at the beginning?

Due to the shape of the capillary tube, and the intensity of the photoacoustic signal generated by the positive control, a significant ringdown effect occurred, which resulted in signal that were brighter at the end of the acquisition. For data analysis and image reconstructions, the range of time (2 μs) corresponding to the position of the actual capillary tube was utilized.

Discussion

*** Please discuss the limitations and challenges of this method looking towards clinical translation. For example, the ability of the system to detect CTCs through tissue and whole blood. Or perhaps the usage of nanoparticles for a clinical screening exam. Or the length of time a patient would need to be monitored to detect a CTC?

We have included the following section to address translation of this method into clinical settings.

> *"Future validation of this method will include: testing with human clinical samples, implementation of high throughput testing and analysis, and translation of this tool into clinical settings.."*

Significance of the method with respect to existing/alternative methods: This is only vaguely discussed. In the manuscript the authors state, "PAFC introduces several advantages over these traditional methods for the detection of CTCs, including the ability to detect CTCs within patient samples, and its ease of translation to in vivo applications." However, neither of these is shown in the manuscript and therefore more elaboration and citations are required.

The potential advantages of PAFC over traditional methods have been clarified in the discussion section as indicated below.

> *"Our current system is specifically suited for ex-vivo detection of CTCs, however, this method identifies the potential for future application of FA-CuS NPs in vivo.*

Additionally, the authors state, "PAFC has also been shown to accurately detect CTCs in vitro and in vivo when combined with targeted contrast agents" and later "Our results identify the detection of 353 SKOV-3 cells down to a concentration of 1 cell/μL," but this is not shown in the manuscript with the representative results as is, instead the representative results depict signals from a solution of nanoparticles only, not cells incubated with nanoparticles." These statements need to be clarified and expanded upon.

We thank the reviewer for their comments. A reference to our previous work has been added.

> *"Our results identify the detection of SKOV-3 cells down to a concentration of 1 cell/μL.[1]"*
>
> 1. Lusk, Joel F., et al. "Photoacoustic Flow System for the Detection of Ovarian Circulating Tumor Cells Utilizing Copper Sulfide Nanoparticles." *ACS Biomaterials Science & Engineering* 5.3 (2019): 1553-1560.

Response to Reviewer 2:

Manuscript Summary:

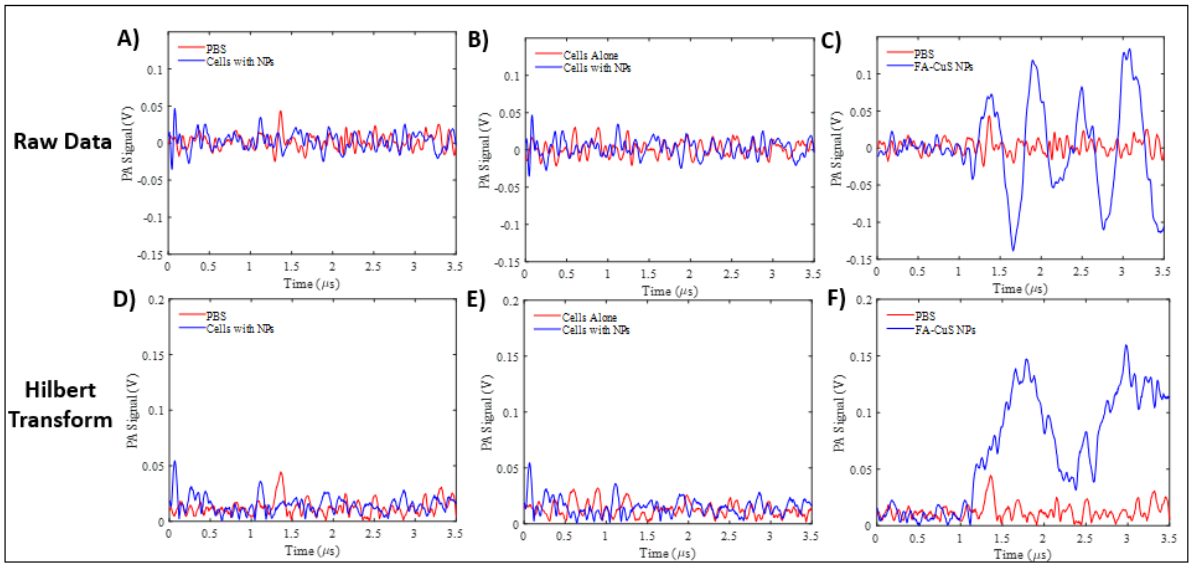
The table of materials still lists the transducer as made by Master Flex - shouldn't it be Olympus? Otherwise, the authors have made satisfactory revisions and I suggest the manuscript is ready for publication.

We thank Reviewer 2 for their feedback. The materials list has been corrected to address your concerns. We have addressed each of your questions included below. Our responses have been made in green, so that it is easy to reference, and corresponding updates to the manuscript are also in green. We have broken up each of the comments into smaller segments and have addressed them individually.

A few comments for future work that don't really impact this manuscript:

1) The y-axis scales of fig. 4 are deceptive. A/B/D/E are -0.05 to 0.05, while C/F are higher. At first glance, it seems that the PA signal is not much greater than the noise. In the future, using the same scale length for all would emphasize the stronger PA signal compared to noise.

We thank the reviewer for their observations regarding the y-axis scales of fig. 4. These corrections have been added to the manuscript and included below:



2) I'm puzzled about the hardware used -- a 35 MHz preamp was used with a 50 MHz transducer to detect signals <20 MHz. This doesn't make sense to me. Why were the signals at <20 MHz? The sensitivity of the 50 MHz at these low signals is very small, why not just use a lower frequency transducer?

We have updated the manuscript to acknowledge the reviewer's comments:

> *"For our current PAFC system, preliminary studies confirmed photoacoustic detection using the transducer and amplification system. The majority of these signals are comprised of lower frequency signals (<20 MHz). Further studies are needed to confirm whether this is due to the actual frequency of the generated photoacoustic signals or the 35 MHz bandwidth of the amplifier. Future studies will investigate the frequency components of the detected signals in order to optimize the central frequency of the transducer as well as the bandwidth of the amplification system."*

3) The throughput is extremely low at <10 samples/minute. CTCs tend to be quite rare, and take impossibly long using this system. The system would have to be sped up by orders of magnitude to do ex vivo CTC diagnostics within a reasonable timeframe.

The authors have addressed the reviewer's comments in the manuscript in the following passage:

> *"Future validation of this method will include: testing with human clinical samples, implementation of high throughput testing and analysis, and translation of this tool into clinical settings.."*

4) The concept of measuring PA signals from "plugs" in vivo is just not possible -- blood cannot be split like that. How will it be done? I envision a form more like Zharov et al, which is already in clinical trials.

The manuscript has been updated to better clarify clinical application of the PAFC system.

> *"The current system is specifically suited for ex-vivo detection of CTCs, however, this study identifies the potential for future application of FA-CuS NPs in vivo."*

Response to Reviewer 3:

Manuscript Summary:

> We thank Reviewer 3 for the feedback. We have addressed each one of your questions included below. Our responses have been made in orange, so that it is easy to reference, and corresponding updates to the manuscript are also in orange. We have broken up each of the comments into smaller segments and have addressed them individually.

General Comments:

While I'm happy with the corrections made to the protocol, I'm still a little concerned about the argument shown in L434-436. You cannot make the claim that your signals were mostly <20 MHz considering the sensitivity of the pre-amp, i.e. energy at this frequencies was possible, just that your system couldn't detect them. Could this just be rephrased to say to acknowledge this combination of transducer/pre-amp might not be optimal?

We have updated the manuscript to address the reviewer's comments as indicated below:

> *"For our current PAFC system, preliminary studies confirmed photoacoustic detection using the transducer and amplification system. The majority of these signals are comprised of lower frequency signals (<20 MHz). Further studies are needed to confirm whether this is due to the actual frequency of the generated photoacoustic signals or the 35 MHz bandwidth of the amplifier. Future studies will investigate the frequency components of the detected signals in order to optimize the central frequency of the transducer as well as the bandwidth of the amplification system."*

Editorial comments:

Please revise the reference format to be [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage (YEAR).]

We have revised our reference format to be consistent with the JoVE template, and replaced citations that were placed incorrectly in the bibliography.