APPROVAL NOTICE

DATE:	15 Nov 2024
TO:	Ekaterina Galanzha, PhD
PROTOCOL:	CytoAstra, LLC, In vivo liquid biopsy for early detection of metastatic melanoma (Pro00075507)
APPROVAL DATE:	12 Nov 2024

IRB APPROVED:

Documentation:

- Protocol (Not Dated)

The IRB reviewed and approved the above referenced documentation submitted on your behalf.

The IRB determined there were no changes required to the current Consent Template(s).

It is the IRB's expectation that any placeholders in approved subject-facing material(s) will be accurately populated with the IRB approved content in all applicable instances.

Audio/visual recruitment or subject material approved in script format only must be submitted in final format for the IRB to review what potential subjects will see or hear. The IRB does not review the content found in embedded links or QR codes, therefore this content must be submitted for review and approval separately, prior to use.

If you wish to appeal the IRB's determinations and/or imposed modifications, please submit supporting documentation to address the IRB's concerns by creating an Appeal Modification in CIRBI.

Compliance Statement/REB Attestation (Applicable for research conducted in Canada):

The IRB attests that this submission has been approved by an IRB whose membership complies with the requirements defined in Health Canada regulations, ICH GCP guidelines, FDA regulations at 21 CFR part 56, and HHS regulations at 45 CFR part 46. The IRB carries out its functions in accordance with FDA regulations at 21 CFR parts 50, 56, 312, and 812; HHS regulations at 45 CFR part 46, subparts A-E; good clinical practices; Health Canada regulations; and the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans, as appropriate to the research.

Advarra IRB is registered with OHRP and FDA under IRB#00000971.

Please review the IRB Handbook located in the "Reference Materials" section of the Advarra CIRBI™ Platform (www.cirbi.net). A copy of the most recent IRB roster is also available.

Thank you for continuing to use Advarra IRB to provide oversight for your research project.

Sincerely,

Luke Gelinas, PhD
Executive Board Chair

Study Title: In vivo liquid biopsy for early detection of metastatic melanoma

Proposed dates for data collection:

Start date: TBD

Institution/Sponsor: CytoAstra LLC

I certify that the information provided in this application is complete and correct. I understand that as principal investigator (researcher), I have ultimate responsibility for the conduct of the study, adherence to ethical standards, and protection of the rights and welfare of human participants. I agree to: (1) conduct the study according to the approved protocol; (2) make no changes to the approved study without prior IRB approval; (3) use the approved procedure and form(s) for obtaining informed consent; and, (4) promptly report any significant adverse events to the IRB within five working days of occurrence.

Researcher's Name	Date
Advisor/Mentor/Supervisor/Sponsor Name	Date

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Abbreviations

AE	- Adverse event
AJCC	- American Joint Committee on Cancer
CCTRA	- Cancer clinical trials and regulatory affairs
cfDNA	- cell-free DNA
CT	- Computer tomography
CTC	- Circulating tumor cell
ctDNA	- circulating tumor DNA
DNA	- Deoxyribonucleic acid
DOB	- Date of birthday
FDA	- Food and Drug Administration
HIPAA	- Health insurance portability and accountability act
Hz	- Hertz
ID	- Identification
IDE	- Investigational device exemption
In vivo	- Latin for “in a living organism”
IRB	- Institutional review board
kHz	- Kilo-hertz
LLC	- Limited liability company
MEM	- Error on mean
MRI	- Magnetic resonance imaging
μJ	- Microjoule
mL	- Milliliter
nm	- Nanometer
ns	- Nanosecond
NSR	- Non-significant risk
OS	- Overall survival
PA	- Photoacoustic
PAFC	- Photoacoustic flow cytometry
PET	- Positron emission tomography
PFS	- Progression free survival
PHI	- Protected health information

PI	- Principle investigator
RNA	- Ribonucleic acid
SLN	- Sentinel lymph node
TBD	- To be determined
US	- United States

Study Summary (Goal, Hypothesis and Objectives)

The majority of melanoma deaths are associated with metastasis that are formed by cancer cells shedding from the primary tumor, enter the circulation and spread to distant organs via the blood.[1-2] These cells in blood are termed circulating tumor cells (CTCs). Growing evidence indicates that metastasis is an early event in melanoma patients, often occurring even before metastases are clinically detectable.[1,3] Therefore, detection of CTCs using liquid biopsy assays should be considered as effective tools to early diagnose a risk of metastasis progression.[1-2, 4-5] Taking into account the link of CTCs with metastasis, and thus melanoma aggressiveness, CTCs have an advantage over many other biomarkers in identifying patients for early therapeutic intervention at a stage when the disease is still curable. This advantage might also help to avoid underdiagnosis, which is a major issue in melanoma. Overall, despite CTCs spread occurs early, they are usually currently detected late.[1]

Currently, the main issue restricting the use of CTCs for early melanoma detection relates to the low sensitivity in the detection of CTCs in conventional small blood samples.[1,6-7] Clearly, it is important to maximize the blood volume available for analysis. It can be achieved by in vivo CTC assessment. Toward this goal, in vivo CTC enumeration without labelling in a large blood volume using photoacoustic (PA) flow cytometry (PAFC) diagnostic platform was developed by our team.[6] Clinical testing of this technology revealed a detection sensitivity of 1 CTC per liter of blood (~1,000 times better than that of existing assays), with CTCs being detected in 27 (sensitivity 96.4%) of 28 patients with melanoma and 0 of 19 individuals without cancer (specificity 100%).[7]

The **Goal** of this clinical study is to determine whether a PAFC device called the Cytophone can be used for in vivo detection and enumeration of circulating tumor cells (CTCs) in the melanoma patients at different disease stages with focus on early diagnosis, early assessment of disease recurrence and monitoring of therapy efficiency. It is expected that the detection limit of CTCs will be improved at least an order of magnitude compared to the detection limits of existing methods.

We **hypothesize** that PAFC-based CTC assay in vivo provides earlier, rapid and more accurate prognosis of metastasis progression, disease recurrence and therapy efficiency.

To achieve our goal, we will accomplish the following **primary and secondary objectives**:

<u>The primary objectives</u>:

1. Obtain evidence that positive PAFC-based CTC test indicate a risk of metastasis development
2. Define thresholds of CTC counts that correlate with melanoma recurrence and progression of metastatic disease and therapy efficacy

<u>The secondary objective</u>:

1. Determine if PAFC diagnoses risk of melanoma metastasis progression and recurrence earlier than existing methods.

Background

Worldwide, more than 350,000 people are diagnosed with melanoma and nearly 60,000 deaths are attributed to melanoma annually.[8] In the US, melanoma is the fifth most common cancer (https://www.cancer.net/cancer-types/melanoma/statistics) resulting in a high mortality rate. According to the American Cancer Society (https://www.cancer.org/cancer/melanoma-skin-cancer/about/key-statistics.html), the estimates for melanoma in the US for 2023 is about 97,610 new cases, and about 7,990 people are expected to die of melanoma. The main cause of melanoma deaths is metastasis.[1,8-9] Indeed, the localized primary tumors can be effectively treated, but if tumor cells detach from primary tumor, prognosis worsens, and treatment becomes much less effective. As an example, the 5-year relative survival rate in patients with "thin melanoma," with no detectable metastasis is 99% while it dropped to 32% for patients with metastatic melanomas.[9]

Despite remarkable progress which has been made on both the diagnosis and therapy of metastatic melanoma, the incidence, recurrence, and death rates remain unacceptably high.[9]

It is important that **(1)** a median delay of early melanoma diagnosis is estimated at approximately 9 months;[8,10] and **(2)** many people still don't benefit from the newest drugs because of melanoma recurrence after initial successful treatment.[11] **Thus, new diagnostic tools to identify early melanoma, especially, its risk to metastasis progression and risk of recurrence in melanoma patients are of paramount importance for surveillance and treatment plans.**

Based on our comprehensive literature review, diagnostic challenges that need resolved are outlined below.

Early detection of a melanoma is a major concern for the clinicians because the existing methods such as biopsy and non-invasive imaging, which are approved in medical practice, are limited to detect early-stage melanoma and risk of its recurrence before the onset of symptomatic metastases already exist.[1,4,9-11] Among many methods, blood melanoma biomarkers (e.g., CTCs, and ctDNA) holds promise to solve diagnostic challenges because CTC detection has potential for pinpointing patients who are prone to metastasis.[4,12-17] As the evidence, multiple clinical studies has been demonstrated that CTC count is associated with risk of disease recurrence and progression free survival (PFS) and overall survival (OS).[2,5,13,15-17] Furthermore, our preclinical study with mouse models showed that melanoma CTCs were detectable soon after formation of primary tumor and before histologically detectable metastasis.[3,18-19] Together, these data show that CTCs have the potential to be used in the early detection and/or diagnosis of recurrence of melanoma. However, there is no consensus between physicians on the role of blood tests in melanoma diagnosis and follow-up.[2,20] This is because there are critical limitations of existing CTC tests. The main drawback of all existing CTC assays is their low sensitivity due to the in vitro methodology.[1,3,7] Specifically, examination of a small volume blood sample (≤10 ml) prevents detection of CTCs in the other 4,990-ml of a ~5-liter total human blood volume.[3,7] Since an early-stage tumor is expected to produce very small number of CTCs (1–3 CTC or less/~5 billion blood cells, current methods lack early CTCs that prevents detection/prognosis of early metastasis.[1,3,6-7,18] Other limitations of CTC methods include low throughput, which require many hours to assess a blood sample in vitro; and multiple sample-processing steps, which result in a 60% to 97% loss of CTCs.[1]

In recent years, the efforts have been made to develop new liquid biomarkers such as cell-free nucleic acids including cell-free DNA (cfDNA), ctDNA and cell-free microRNA.[2,4,17,] It has been found that some CTC-derived mRNA and ctDNA were indicative of clinical outcomes for patients with stage IV melanoma[2] but usefulness of these assays is limited by unknown the origin of

detected nucleic acids. From the use of existing tests, it is not clear whether the ctDNA is actively released from live cells or derived from cells that have undergone apoptosis/necrosis.[2] Furthermore, to distinguish ctDNA from cfDNA, specific somatic mutations in the melanoma tumor must first be identified, but this is sometimes challenging in obtaining sufficient tumor material for genetic investigation within the clinical setting.[13,21-22] This is because some tumors can only be accessed through invasive procedures that provide insufficient genotyping material.

To fill this a gap in early melanoma diagnosis, designing more sensitive and accurate CTC diagnostic tool is in high demand. The ideal method should be highly specific and sensitive to identify patients who are at high-risk of metastasis progression and recurrence after treatment, provide treatment outcomes to personalize systemic treatment, provide prognostic data before, during and after treatment, and monitor response to treatment. Based on our successful preclinical and recent clinical studies,[3,7,18-19] we demonstrated that the PAFC diagnostic platform could be part of the solution (**Fig.1**). In PAFC, the principle of PA detection, coupled with using melanin as an intrinsic marker, to which PA methods are very sensitive (**Fig. 2**). In particular, our clinical trials with PAFC technology revealed a detection limit down to 1 CTC per a liter of blood (~1,000 times better than that of existing CTC assays), with CTCs being detected in 27 (i.e., sensitivity 96.4%) of 28 patients with melanoma and 0 of 19 individuals without cancer (i.e., specificity, 100%) (**Fig. 3**).[7] Based on the obtained data, PAFC advantages include:

- In vivo monitoring of circulating blood to achieve assessment of CTCs in up to one liter of blood volume.
- High throughout because initial diagnostic results such as POSITIVE/NEGATIVE CTC test will be obtained during the monitoring procedure without additional processing steps.
- Recorded and software-treated results will be used to receive extended diagnostic information on CTC including CTC count per a time unit.
- Preventing the potential loss of CTCs because The Cytophone's diagnosis does not require multi-step processing procedures.
- High specificity and sensitivity of PAFC device in detection of melanoma CTCs.
- Reducing toxicity concern because detection procedure is noninvasive and label free: well-known high PA contrasts of intrinsic melanin in CTCs provides label-free diagnosis without injection of any reagents in blood circulation, and in vivo approach prevents blood collection and makes procedure completely noninvasive.

We anticipated that CTCs detected by PAFC will be the first indicators of the early melanoma cell spreading through blood system with the risk of metastasis development. The Cytophone would enable monitoring for this process in a noninvasive manner and have the potential to be applied as a tool for the early detection of metastatically aggressive melanomas. Also, it can support clinicians in making individual (personalized, tailored) plans of treatments and determining who should receive adjuvant therapy (e.g., immunotherapy) resulting in personalized medicine. This decision can help to prevent metastatic progression by early initiation of personalized therapies and minimize the number of patients exposed to treatment-related toxicities that eventually improve patient survival. In addition, PAFC detection procedure would not only improve prognosis and therapy but would ultimately reduce the costs by preventing unnecessary biopsies, screening, and reducing treatment costs. The additional benefits of the Cytophone technology include the expected access to patients (as a mobile-like device) with very low to no challenges in physician training. It should be emphasized that PAFC is not intended to replace the well-

established clinical diagnoses. We anticipated that PAFC would supplement histopathological and imaging diagnosis.

In future, we expect that PAFC technology would have significant advances in using CTCs as excellent marker for screening population to identify asymptomatic individuals with high-risk of metastatic melanoma.

Study Design / Procedures Involved

Start Date: TBD	End Date: TBD

This study will be conducted the Cytophone device for subjects with diagnosed melanoma. Participants need to be 18 years of age or older. The study is not intended to change routine diagnosis and therapy of melanoma patients. Eligible participants will be given the Cytophone's procedure in person after signing the Informed Consent Form and verification of the subject ID, date of birthday (DOB) and name. The blood samples will be collected before a the Cytophone's procedure to detect CTCs and ctDNA using conventional methods *in vitro*. Blood samples will be obtained in the laboratory by a nurse and transferred to the companies for testing melanoma in vitro: Menarini (CTC test) and Billion to One (ctDNA test). Results will be provided by the companies in a few weeks.

It is anticipated that PAFC diagnostic procedure will take approximately thirty (30) minutes including ultrasound imaging of an examined vessel, photo of a skin area above the vessel and quick navigation of PAFC probe on the vessel (**Fig. 3, top**). At early disease stage, the diagnostic procedure can be increased to one hour to test larger blood volume and get more statistical data.

Subjects will come into the assigned clinical room for up to 5 visits. A withdrawal visit will be scheduled as needed. A post observational (medical records, OS and PFS) study design will be implemented to define progression and recurrence of metastasis.

Participants will be screened for any contraindications (physical exam and lab work) to taking the included investigational procedure and evaluated per protocol inclusion/exclusion criteria. The risk to subjects in this trial will be minimal by compliance with the inclusion/exclusion criteria, regular clinical monitoring, avoidance of unnecessary procedures, and adherence to investigator guidance regarding specific safety areas. If, during the study, a female participant becomes pregnant or decides to attempt to become pregnant, then she must stop the study and undergo the rapid exclusion procedure. Female patients must not be breastfeeding or pregnant (as confirmed by serum pregnancy test) at the time of study entry and must agree to undergo serum pregnancy testing throughout the study at each clinic visit. In addition, a serum pregnancy testing should be conducted in case of an unexpected delay of menorrhea.

Location of the study TBD

Groups of the study. All study participants will be blinded from their results. There will be four groups to the study. Subjects will be assigned to study cohorts based on the AJCC Melanoma of the Skin Disease Staging (8^{th} edition) as documented at the time of consent based on current or previous disease status.

1. Group 1: The Cytophone testing of healthy subjects (30 individuals). This group will consist of thirty healthy subjects with all of light skin tones and ten of whom will be of dark skin tones according to the Fitzpatrick classification scale. Subjects will have two visits: one visit for checking eligibility + signing a consent form and one visit for The Cytophone's testing. The obtained data will be used to address the calibration goal. Healthy subjects will be recruited by the assigned clinics based on the willing to volunteer for this group. Clinicians or assigned clinical coordinator will inform the potential subject of the study and refer them to the study team for more information.

2. Group 2: The Cytophone testing of 50 subjects who have been pathologically diagnosed with Stage 0-I melanoma. The initial PAFC testing will occur within 30 days of diagnosis. Subjects will have 4 visits. The first visit will include checking eligibility + signing a consent form, blood draw and the Cytophone's testing. The follow-up three visits (visits 2, 3 and 4) will include the Cytophone's testing. Group 2 participants will be asked to provide their ID number in order to link their in vivo CTC count with medical records including biopsy. The obtained data will be used to link the presence of CTCs in blood circulation or increase in CTC count with metastatic disease initiation.

3. Group 3: The Cytophone testing of 50 subjects who have been pathologically diagnosed with Stage II melanoma. The initial PAFC testing will occur within 30 days of diagnosis. Subjects will have 4 visits. The first visit will include checking eligibility + signing a consent form, blood draw and the Cytophone's testing. The follow-up three visits (visits 2, 3 and 4) will include the Cytophone's testing. An increase in CTC count will possibly demonstrate an early sign of initiation of disease progression. Group 3 participants will be asked to provide their ID number in order to link their in vivo CTC count with medical records including dermoscopy, biopsy, sentinel lymph node (SLN) status, CT, MRI, PET and others. The results will be used to analyze the correlation between CTC count and metastatic disease initiation/progression and further recurrence induced by metastasis-originated CTCs.

4. Group 4: The Cytophone testing of 50 subjects who have advanced-stage melanoma (stages III - IV) with metastatic disease. Subjects will have 4 visits. The first visit will include checking eligibility + signing a consent form, blood draw and the Cytophone's testing. The follow-up three visits (visits 2, 3 and 4) will include the Cytophone's testing. Dynamic of CTC counts will be defined over disease progression and treatment to link CTC counts, metastatic disease progression and therapy efficiency. Group 4 participants will be asked to provide their ID number in order to link their in vivo CTC count with medical records.

Visits. The study will last less than two years and will includes 4 visits, and 12 months follow-up observation of medical records, OS and PFS after 4th visit.

Visit 1 (90 minutes) – at any time in Group 1, after the diagnosis of melanoma in Groups 2-4. The visit will start with checking inclusion and exclusion criteria, confirming eligibility, and obtaining the informed consent. **Only subjects who are eligible and have signed off on the consent form will participate in this study**. Next, the visit will involve blood collection, ultrasound imaging, adjustment PAFC laser beam on the vessel and PAFC monitoring. Also, information on possible adverse events and review of medications and diagnostic tests will be collected, and vital signs (temperature, pulse, blood pressure) will be measured. A pregnancy test will be done if the subject is a female of childbearing potential.

Visit 2 (60 minutes) – in ~90 days after first visit (Groups 2-4). The visit will involve ultrasound imaging, adjustment PAFC laser beam on the vessel and PAFC monitoring. Also, information on possible adverse events and review of medications and diagnostic tests will be collected, and vital signs (temperature, pulse, blood pressure) will be measured. A pregnancy test will be done if the subject is a female of childbearing potential.

Visit 3 (60 minutes) – in ~90 days after second visit (Groups 2-4). The visit will involve ultrasound imaging, adjustment PAFC laser beam on the vessel and PAFC monitoring. Also, information on possible adverse events and review of medications and diagnostic tests will be collected, and vital

signs (temperature, pulse, blood pressure) will be measured. A pregnancy test will be done if the subject is a female of childbearing potential.

Visit 4 (60 minutes) – in ~90 days after third visit (Groups 2-4). The visit will involve ultrasound imaging, adjustment PAFC laser beam on the vessel and PAFC monitoring. Also, information on possible adverse events and review of medications and diagnostic tests will be collected, and vital signs (temperature, pulse, blood pressure) will be measured. A pregnancy test will be done if the subject is a female of childbearing potential.

Follow-up observation (no visit requires) – in 12 months after 4th visit (Groups 2-4).

Procedures.

Investigational PAFC Procedure. At the beginning of each visit, for approximately a few minutes, the investigator will adjust the position of laser beam on the vessel. The investigator will use near-infrared viewer and skin marker. After completion, the Cytophone monitoring of this vessel will be conducted approximately 30 minutes and one hour if needed. The section below provides more details of The Cytophone device and related procedures.

Standard of care procedures

Specimen Handling (Groups 2-4).

A blood draw with a volume of 25 mL will occur in the blood laboratory at the site of melanoma testing via venipuncture standard practices. The sample will be coded using the study subject ID. A 10 mL will be transported to Menarini Silicon Biosystems, Inc. laboratory for detection of CTCs in vitro. Another 10 mL will be delivered to Billion to One for ctDNA testing. Pulse, temperature, height/weight, blood pressure, and pulse will be measured in the sitting position for all subjects.

Pregnancy testing: serum pregnancy tests will be carried out for women of child-bearing potential.

Collected data.

Demographic data, diagnosis, medical records, and medications will be collected at the first visit (pre-visit). Medical records, and medications will be continuing to monitor over a period of 4 clinic visits (~ 9 months) and a post-12-month observation.

The Cytophone records will be use to:

- Collect background signals in vivo and in vitro to estimate rate of false positive signals (Croup 1)
- Calculate number of PA signals per a time unit that are associated with single CTCs and CTC clusters (Groups 2-4)

At the end of the monitoring and observation period, the investigators will analyze the results and determine if the CTC counts correlate with metastatic disease progression and may be used as diagnostic and/or prognostic criterium of aggressiveness of early melanoma, initiation/progression of metastasis, initiation/progression of recurrence and assessment of therapy efficacy.

Photographing Participants.

Participants will be asked a yes or no question on the informed consent as to whether they agree or not to allow the research staff to take an ultrasound images and photo during testing for presentation purposes ONLY. They will be no identifying facial pictures taken.

Study Calendar

	Visit 1; Day 0	Visit 2; Month 3 (± 14 Days)	Visit 3; Month 6 (± 14 Days)	Visit 4; Month 9 (± 14 Days)	Observation of medical records, OS and PFS; Month 24 (± 14 Days)
Group 1					
Informed consent	X				
Inclusion/Exclusion criteria	X				
Vital signs/pregnancy test	X				
PAFC	X				
Photography	X				
Blood sampling					
Review AEs / Medications / Medical records	X				
Group 2-4					
Informed consent	X				
Inclusion/Exclusion criteria	X				
Vital signs/pregnancy test	X	X	X	X	
PAFC	X	X	X	X	
Photography	X	X	X		
Blood sampling	X				

Review AEs / Medications / Medical records	X	X	X	X	X

Description of New Device and monitoring procedure

The Cytophone device is based on a noninvasive, label-free PAFC diagnostic platform (**Fig. 2**). The Cytophone will be used to monitor in vivo (1) healthy volunteers to optimize The Cytophone's parameters and estimate false signal rates, and (2) melanoma patients to identify the presence of melanoma CTCs and their clusters (aggregates, clots), and to enumerate them per a time unit.

The PAFC/The Cytophone clinical prototype incorporates pulsed two pulse lasers, optical system for delivery of laser light to the skin, ultrasound transducer array and detection electronics for acquisition of acoustic waves from CTCs (**Fig. 4**). Parameters of lasers:

C-Wedge 1064 (Bright Solutions, Cura Carpignano (PV), Italy):
Wavelength,1064 nm; pulse width, 0.6 ns; pulse rate: 1 kHz; pulse energy, 240 μJ.
C-Wedge 18901 (Bright Solutions, Cura Carpignano (PV), Italy):
Wavelength, 770 nm; pulse width 3 ns; pulse rate, 1 kHz; pulse energy, 240 μJ.

The lasers complement each other when in use, if there is a failure or downtime with one of the lasers, then the operational laser can still be used monitoring patients.

Detection of acoustic waves from CTCs is performing by focused ultrasound transducer array placed in an acoustic contact with the skin of the subject with ultrasound gel. The signals from the transducers are amplified with 16 channel amplifier (**Fig. 4**), and then digitized by a 12-bit, 500 MSPS high speed board digitizer (ATS9350, AlazarTech, Canada) and transfer to data recording system using a laptop. The PAFC platform is assembled on a cart. The laser body, computer, and all the accessories are self-contained within the cart. Internal components of the setup are not accessible to the subject. The top of the cart contains the PAFC probe on a thin optical plate. A manual stage is used to provide positioning of the probe over the vein of the backhand, wrist or forearm. Delivery of the laser light to the probe is performed by an optical fiber or by lens optics. Positions of the ultrasound transducer and optical fiber or lens will be fixed after a short initial probe alignment procedure.

Subjects will receive PAFC procedure while seated in a chair. The subject's hand will be placed on a customized surface, with a PAFC probe placed over a selected vein. Acoustic contact between the skin and ultrasound transducer will be provided by a gel. Flexible Velcro strips is used to provide gentle fixation of the hand during PAFC monitoring. PAFC will be used to monitor blood flow during 30 min. If necessary (e.g., due to signal instability or low signal levels) this procedure will be repeated on a different blood vessel(s) up to an additional 30 minutes. PA measurements will be performed the entire time the subject holds the hand under the probe. The position of the probe over the vein will be monitored, if necessary, by comparing the signals from blood vessel to that from surrounding tissues and may require adjustment during the experiment.

While the device is contained on a cart, it remains in one location, except when maintenance requires moving it to the company. If the device is moved for maintenance, it is recalibrated after returning it to the clinical site.

Subject Population and Inclusion/Exclusion Criteria

Study Population: To achieve our goal, we expect to enroll 180 participants (18-80 years old) during the proposed timeframe. Males and females will be eligible for this study. No exclusions will be made regarding ethnicity. The target is to enroll 20-25% of subjects with dark skin.

Prior to coming in for the Cytophone's testing, subjects will sign the consent and complete a medical history questionnaire.

Group 1: 30 healthy subjects, twenty of whom will be of light skin tones and ten of which will be of dark skin tones according to the Fitzpatrick classification scale.

Groups 2-4: 150 subjects (50 subjects per a group) with melanoma will be assigned to study based on the AJCC Melanoma of the Skin Disease Staging (8^{th} edition) as documented at the time of consent based on current or previous disease status.

All participants will receive 4 PAFC procedures over ~9 months. A blood sample will be taken for in vitro CTC and ctDNA tests.

Inclusion and Exclusion Criteria

Inclusion Criteria for Group 1 (Healthy Subjects):

- Aged 18–80 years, inclusive
- Free of concomitant or previous malignancies, as deemed by the Principal Investigator, is not contraindicated with any past medical history of the participant
- Participants having provided informed consent with signature on informed consent form: the informed consent process should be complete with full discussion of all requirements and possible risks.
- Must be able to sit for up to 60 minutes

Inclusion Criteria for Groups 2-4 (Melanoma Patients):

- Aged 18–80 years, inclusive
- Histological documented diagnosis of melanoma
- Participants having provided informed consent with signature on informed consent form: the informed consent process should be complete with full discussion of all requirements and possible risks.
- Must be able to sit for up to 60 minutes

Exclusion Criteria:

- Unable to provide informed consent to participate in the study, such as a mental condition rendering the participant unable to understand the nature, scope, and possible consequences of the study
- Clinically relevant cardiovascular, hepatic, neurological (e.g., evidence of organic brain syndrome), endocrine, or other major systemic disease making implementation of the protocol or interpretation of the study results difficult or that would put the participant at risk by participating in the study
- Persistent significant or severe infection, either acute or chronic
- Pregnant or breast-feeding women or those who plan to become pregnant during the study

- Women of childbearing potential not protected by effective contraceptive method of birth control and/or who are unwilling or unable to be tested for pregnancy.
- Any known history of severe preexisting constipation

Research staff will review this list of exclusion criteria with each participant at the time of consent in order to identify eligibility.

Recruitment Methods

The study and recruitment information will be also available to all interested participants on the CytoAstra.

Investigator contact information will be included in all advertisement materials for additional questions or concerns regarding the study.

Subjects who are interested will be asked to contact the study team to complete a prescreening. Research staff has been trained on enrollment of patients and Cytophone operation.

The PI and research assistants will be responsible for recruitment, retention and coordination of subjects.

After signing the IRB-approved informed consent form, research subjects will be registered on the clinical site performing the Cytophone test. At that time, a registration number will be provided to the Principal Investigator and research staff. The initial procedure must be conducted within 90 days of consent, or the subject will be withdrawn from the study and will need to re-consent in order to participate. Subjects who are withdrawn from the study due to delay of procedure may be replaced.

All these forms clearly indicate the participation in the study is voluntary.

Consent Methods

The informed consent form will be presented to participants at the first visit. The consent process will occur in a private room with the research nurse. A research member will explain the informed consent form for each potential participant. All participants will be given the option to ask questions, they will be given ample time to consider participation and the benefits and risks associated with that participation. The participants will be asked to sign the informed consent upon agreement of the terms and conditions associated with the study. Participants will also indicate that they have read the consent form and agree to participate by selecting 'yes' on the consent form page.

Participants can withdraw from the study at any time without any consequences.

Signed consent documents returned via fax, email, or mail should be accompanied by copies of official documentation identifying the potential subject and witness. Consent documents returned by any of these methods will be signed/dated by study staff with the current date. The consent process will be documented in the medical record. The original informed consent will be filed with the subject file. Each participant will be given a copy of the informed consent.

Potential Risk/Benefits to Subjects or Others

Risks.

Minimal risks are expected because the Cytophone (PAFC) device with the same lasers and the same parameters was approved by the IRB committee at the University of Arkansas for Medical Science (Protocol #133965) and the FDA classified this device as a Non-Significant Risk (NSR) medical device with the Investigational Device Exemption (IDE). Moreover, clinical testing of melanoma patients in vivo using PAFC with the same parameters revealed no adverse effects.[7] Thus, The Cytophone is regarded as safe but may cause:

- Local skin irritation and redness as well as burning, tingling, itching, stinging or localized pain sensations; careful monitoring of skin changes will be provided and local treatment including ice pack, skin lotion or topical steroid will be provided as needed.
- Discomfort during sitting for the time required to complete the procedure; the participant may request that the procedure be stopped at any time.

Risks associated with blood draws may include bruising, minor pain and rare infection at the puncture site. Experienced personnel will perform this procedure using approved techniques. Pressure and dressings will be used to minimize pain, bruising and infection.

There is also a minimal risk associated with loss of confidentiality or anonymity. To minimize this risk, participants' identity will be kept confidential at all times by the research team. Subjects will be assigned a study number unrelated to their medical record number or other personally identifying information. The obtained research data will be associated with the subject's number only. The key to the participant identity will be kept in a locked file cabinet behind a locked door that only the research team will have access to. Signed consent forms will also be kept in this locked file cabinet but separate from the data.

No risks to others are anticipated.

Benefits.

While there are no direct benefits to the participants in this study, results may help practitioners to better understand the Cytophone (PAFC) diagnosis and use it in future as an advanced test for early melanoma diagnosis, early detection of disease recurrence, monitoring of therapy efficiency, and potentially melanoma screening.

Safety Endpoints

If any of the participants reveal procedure-related side effects or Adverse Events (AEs), the subject will be withdrawn. If three people are withdrawn for that reason, the study will be stopped.

Withdrawal of Subjects

Subjects will be withdrawn from the study if:

1. The Investigator considers it would be in the best interest of the subject (e.g., AEs, intercurrent illness or onset of new clinically significant condition).
2. Female subject becomes pregnant.
3. There is a protocol violation which increases the risk to the subject.
4. Subject withdraws consent or is lost to follow-up.

The subject will be contacted immediately to schedule a withdrawal visit. If the subject cannot be reached on first attempt, then a second and third attempts to contact the subject via phone and email will be made.

If and when a subject withdraws from the study for any reason, all efforts will be made to complete and follow the participant through observations as thoroughly as possible. A final evaluation will be attempted in those willing to participate.

Provisions to Monitor the Data to Ensure the Safety of Subjects

AEs and safety labs will be reviewed at each visit by the PI. Additionally, subjects will be instructed to call the study team to report new symptoms/AEs. These AEs will be recorded in the subject's binder on a cumulative AE log. If a serious AE is unresolved when a subject permanently discontinues the study, the subject will be followed until the AE resolves, or the clinical course is stabilized.

An independent safety monitoring committee involving a physician (James Suen, MD), engineer (Aayire Yadem, PhD) and bio-statistician (TBD) will review safety labs and AEs after the first 5 subjects are enrolled and then after the 50th and 100th subjects are enrolled. He/she will notify the study team if he determines that the study must stop for safety concerns and will be charged with the responsibility of determining if the trial needs to be stopped based on lab criteria and data from any unexpected AEs. These unexpected AEs will be promptly reported to the independent safety monitoring physician.

The whole study will be stopped in cases where serious AEs are experienced by 3 or more subjects and are not deemed unrelated to participation in the study by the PI or the independent safety monitoring committee. Any AE will be immediately communicated to the PI in writing notifying of the event(s).

Provisions to Protect the Privacy Interests of Subjects

All subject interactions will occur in a private room or lab setting. Subjects will be introduced to the various study team members by the PI or study coordinator with whom they've already established a relationship through prescreening or the consent process.

The research team will obtain consent prior to receiving any information about subjects.

Subject Compensation/Reimbursement

There could be compensation for participating in this study. The compensation would be paid on a per visit basis, and only paid once each visit is completed for the study.

Data Management and Confidentiality

Study data will be submitted according to protocol requirements for all subjects registered.

An EDC, Electronic Data Capture system, will be in place for use by the study coordinator and their team. All documents that can be stored in the EDC will be entered and secured. All data will be stored electronically in the EDC or some other secure storage device. Any external storage devices will be encrypted and under the control and supervision of the research team. Any hard or non-electronic records will be stored securely by the research team. Both electronic and hard copies will be secured only allowing access by the research staff.

Electronic data will be entered into a password protected file. Cytophone's records and blood samples will be coded and labeled with study subject's number. Subject initials, age, or any possibly identifying information will not be included in the code. A code link will be kept in a password protected database separate from the blinded codes. This de-identified information (only using assigned study numbers) will be kept within the password protected server.

All data will be stored for a minimum of 5 years after the study is closed with the IRB. Hard copy data will be destroyed via secure shredding per HIPAA standards. Electronic data will also be permanently deleted. Confidential data and PHI will not be disclosed outside of the study team except as required by law or as allowed by the consent (e.g., with the approved IRB or those who monitor the study).

Participants will be asked a yes or no question on the informed consent as to whether they agree or not to allow the research staff to take a photo during testing for presentation purposes ONLY. Care will be taken to block out identifiable characteristics in the photo such as the participants face if they are willing to allow a photo taken during testing.

Upon collecting the data, the results will be analyzed and documented. All data (completed instrument tools, observation notes, and tape recordings) will be kept confidential. Original data will be stored in a locked cabinet in the researcher's office.

Specifically, baseline and demographic characteristics will be summarized by standard descriptive summaries (e.g., means and standard deviations for continuous variables such as age and percentages for categorical variables such as gender). Non-parametric analyses will be used in situations where the variables are not normally distributed.

CTC counts per a time unit in vivo and in vitro as well as ctDNA concentration at Day 0, Month 3 or after surgery, Month 6, Month 9 and Month 12 will be calculated and averaged with coefficients of variation. The increasing trend of CTC counts will be estimated and correlated with other signs of metastasis initiation/progression.

All subjects entered into the study at the first visit will be included in the safety analysis. The frequencies of AEs by type, body system and severity will be summarized. Severe AEs will be described in detail.

Identifiers collected will be entered directly into a prescreening form, which is password protected. If the potential subject fails prescreening, identifiers will not be collected. If the potential subject declines to participate, identifiers will be destroyed upon declination. If the potential subject signs consent, their name and email address will be copied to a screening log in and removed from the prescreening form. Any remaining information on the prescreening form will be permanently deleted from the system when prescreening closes. PHI will not be reused/disclosed to any other

person or entity except as required by law, for authorized oversight of the research, or for other research which use/disclosure of PHI would be permitted by the HIPAA privacy regulations. Identifiable information collected during prescreening (names, email addresses) will only be collected if the potential subject meets the eligibility criteria. Health information will not be recorded during prescreening but will be reviewed to confirm eligibility.

Conflict of Interest

Dr. Galanzha has a proprietary interest in the device, which is associated with CytoAstra, LLC as she is an inventor of PAFC (Cytophone), that have obtained US Patents, 11154360 and 11259705. She is also employed by CytoAstra. To prevent any COI concerns, Dr. Galanzha will NOT have direct contact with human subjects on this study at any time or will not try to recruit subjects for this study.

Drs. Yulian Menyaev, Safi Ullah, and Aayire Yadem have a proprietary interest in the device associated with CytoAstra, LLC. They are currently employed by CytoAstra. They will serve as operators of the device and will only be referred a subject ID when administering the device. Communication will only be limited with the subject to successfully administer the device.

Dissemination of Data

Results of this study may be used for presentations, posters or publications. The publications will not contain any identifiable information that could be linked to a subject and will be reviewed by the Sponsor prior to publication.

Sharing of Results with Subjects

Results will not be shared with subjects.

Data Analysis and Evaluation Techniques

This study will help to develop protocol and standards for in diagnostic CTC test in vivo for using in routine clinical practice for melanoma patients. The obtained results will be published in the peer-reviewed journals, presented at the conferences and used in gaining broader approval of the technology by regulatory agencies. The data analysis will be conducted as outlined below.

***Analysis plan for Group1 (healthy subjects)*:** The study will provide data for the optimization of technical parameters of PA probes that do not require special statistical consideration. Specifically, it will include medium types for acoustic coupling of transducer and skin, method of visualization of laser beam on the skin and vessels, mechanical holder for spatial fixation of PA probe near skin, and signals from blood vessels.

We will define beforehand a window of equivalence with upper and lower limits of equivalence. For example, if we want the two signal amplitudes to be similar to within ±30%, then the window of equivalence would have an "upper limit of equivalence" at +30% and a "lower limit of equivalence" at –30%. Measure 20 blood vessels in the dorsal side of the hands of 3-5 individuals. We can use both hands from the same person. On each vessel, we will use both lasers in second mode for vessels only (background signals). Then we will calculate the difference in signal amplitudes from each laser. We will express the difference as a percentage relative to skin background without visible vessel as well as the percentage relative to Laser 1, or the percentage relative to 0.5*(Laser 1 + Laser 2). Then we will calculate the mean of the percent differences and calculate the 90% confidence interval on that mean. If both ends of the 90% confidence interval fall inside the window of equivalence (i.e., if both ends fall in between the upper and lower limits of equivalence), then you will have established that the two lasers give background signals from vessels only whose signal amplitudes are equivalent to within ±30%.

PA signals from the vessels, PA signals from the surrounding tissues, and PA contrasts will be displayed as dot plots; subjects will be grouped according to skin pigmentation. PA signals and contrasts also will be displayed as scatterplots versus vessel diameter, color-coded by subject. Data will be reported by vessel location and skin pigmentation as the mean, standard deviation (SD), median, quartiles, and range. Additionally, each PA contrast will be evaluated for whether it is "usable" vessel location, then humans. In the secondary post-hoc analysis, we will conduct pairwise comparisons of the skin pigmentations, and likewise of the body locations, using a two-sided 5% alpha despite the multiple comparisons in order not to inflate Type II error. The vessel diameter will be added to the mixed model as a continuous covariate to determine whether larger PA contrasts are associated with larger vessel diameters, and whether including vessel diameter reduces the width of the 90% confidence intervals around the group means.

The defined parameters will be used for further statistical considerations in Groups 2-4.

Analysis plan for Groups 2-4 (melanoma patients): For each melanoma subject, the results of in vitro assay will be classified as a detection or non-detection of CTCs; if the assay result indicates that CTCs were detected, then CTC concentration will also be estimated and classified as above or below the effective lower limit of the standard curve's accuracy. On the same melanoma subject, the in vivo Cytophone's results will be classified as a detection or non-detection of CTCs; if CTCs are detected, then the CTC counts per minute will be converted into a CTC concentration using the blood-flow rate obtained from Doppler ultrasonography on the same vessel region. The discordance rate in CTC detection between in vivo and in vitro method will be determined using paired-data contingency tables. Discordances will then be tested at 5% alpha via one-sided

McNemar's tests for significant imbalance favoring successful detection by the in vivo method. Additionally, whenever the in vivo Cytophone detects CTCs but the in vitro assay does not, the in vivo CTC concentration will be converted via the Poisson distribution into a non-detection probability for the in vitro assay based on the reaction volume utilized by the in vitro method. Finally, whenever CTCs are detected by both in vivo and in vitro method, the paired difference in CTC concentrations between in vivo and in vitro method will be calculated and summarized using the number, mean, and SD.

We will define beforehand a window of equivalence with upper and lower limits of equivalence. For example, if we want the two signal amplitudes from CTCs to be similar to within ±50%, then the window of equivalence would have an "upper limit of equivalence" at +50% and a "lower limit of equivalence" at –50%. We will measure CTCs in blood vessels in the dorsal side of the hands of 5-7 individuals. On each vessel, we will use both lasers. Then we will calculate the difference in signal amplitudes from each laser. We will express the difference as a percentage relative to vessel background as well as the percentage relative to Laser 1, or the percentage relative to 0.5*(Laser 1 + calculate the 90% confidence interval on that mean). If both ends of the 90% confidence interval fall inside the window of equivalence (i.e., if both ends fall in between the upper and lower limits of equivalence), then you will have established that the two lasers give the signals from CTCs only whose signal amplitudes are equivalent to within ±50%.

For in vitro assay, the relevant standard curve will be used to estimate a CTC concentration in the blood of each melanoma subject. On the same subject, the CTC counts per minute obtained from in vivo Cytophone monitoring will be converted into a CTC concentration using the blood-flow rate obtained from Doppler ultrasonography on the same vein region. Data will be transformed to their base-10 logarithms both to reduce heteroscedasticity and to facilitate inference on fold-change differences. Agreement between in vivo and in vitro methods will be assessed using the methods of Bland and Altman. Specifically, for the in vivo method and in vitro method, the average and difference in CTC counts between methods will be calculated for each subject and plotted in a Bland-Altman plot. The 95% limits of agreement between in vivo PAFC and in vitro method will be calculated from the difference in CTC counts as the mean ±2 standard deviations. The mean difference in CTC counts between in vivo and in vitro method will be reported along with a two-sided 95% confidence interval. The mean differences of 5-fold or less will be considered good concordance between in vivo and the in vitro methods, whereas mean differences of 10-fold or more will be considered cause for concern.

Sample size considerations: For the primary analysis of the Group 1, we will use a mixed- models repeated-measures approach to estimate the skin-pigmentation group means and symmetric two-sided 90% confidence intervals for measurements from the hand of each study subject. The half-widths of the confidence intervals (a.k.a. the 90% margins of error on the means, or 90% MEMs) will depend heavily both on the number of subjects in a given skin-pigmentation group and on the intra-subject correlation among individual PA contrasts. **Figure 5** shows how the 90% MEMs vary with intra-subject correlation for exemplar skin-pigmentation group sizes of 3, 5, and 7 per group when the total sample size is 30 healthy volunteers. The 90% MEMs (in SD units) are for the group means of measurements taken from the hands of study subjects. They are calculated using the method of Donner under the assumptions (1) that PA contrasts will be determined on exactly 2 vessels per hand or exactly 3 vessels per hand, and (2) that the denominator degrees of freedom will be 12 based on all three skin-pigmentation groups being represented among the 30 study subjects. Inasmuch as the intra-subject correlation is unlikely to

be >0.5, the range of 90% margins of error shown in **Figure D** indicate that 30 healthy volunteers should provide sufficient precision to meet its research goals. For Groups 2-4, a sample size of 50 melanoma subjects produces a symmetric two-sided 95% confidence interval on the mean difference between methods that has a half-width of 0.258 SD units, where one SD unit is one standard deviation of the difference between methods. In each group, the CTC detection rates via the *in vivo* Cytophone versus the *in vitro* methods will be compared in fifty samples from melanoma subjects via one-sided McNemar's test at 5% alpha to determine whether *in vivo* diagnosis has a significantly increased detection rate. To calculate power of the planned test, we assume that, when the two methods produce a discordant pair of results on the same sample (i.e., when the *in vivo* Cytophone disagrees with the *in vitro* method on whether CTCs have been detected), the odds ratio is 10-fold in favor of *in vivo* data having detected the CTCs. **Figure 6** shows how power to detect the 10-fold odds ratio varies with the proportion of discordant pairs of assay results among a fixed sample size of N=50 melanoma subjects. Since the subjects with a current or recent history of melanoma are expected to have CTCs but have them at concentrations at or below the detection limits of existing *in vitro* assay methods, the proportion of discordant assay-result pairs among subjects should be quite high if *in vivo* Cytophone works as advertised. **Figure 6** demonstrates that 50 subjects should provide sufficient power to meet the proposed research goals.

Data Storage

Individual information will be protected in all data resulting from this study. No personal information will be collected other than basic demographic descriptors. The Cytophone's results will be analyzed and documented. All data (completed instrument tools and observation notes) will be kept confidential. Original data will be kept on a password protected computer, stored in a locked cabinet in the researcher's office and destroyed three years after completion of the research.

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Figures

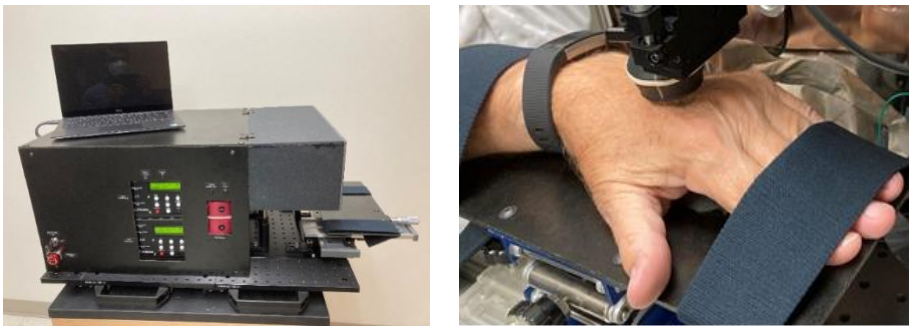

Figure 1. Clinical The Cytophone (left) and PA probe position on the hand (right).

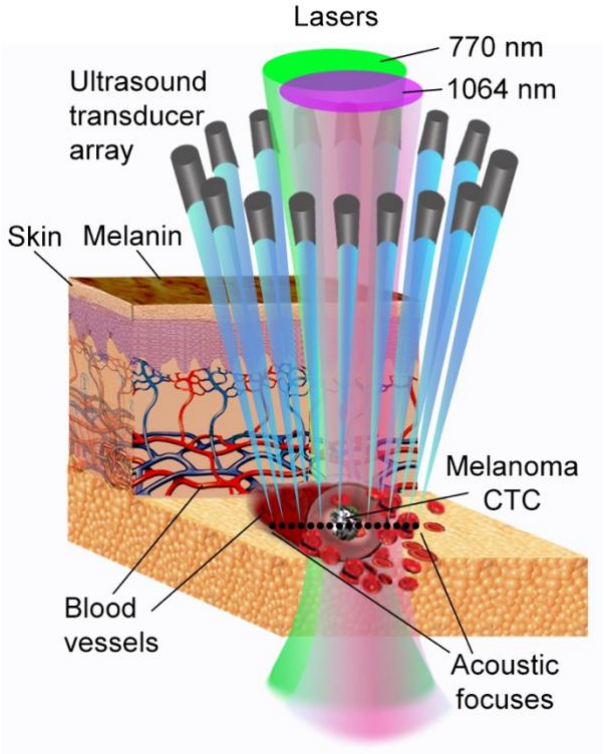

Figure 2. The principle of CTC detection with the Cytophone platform.

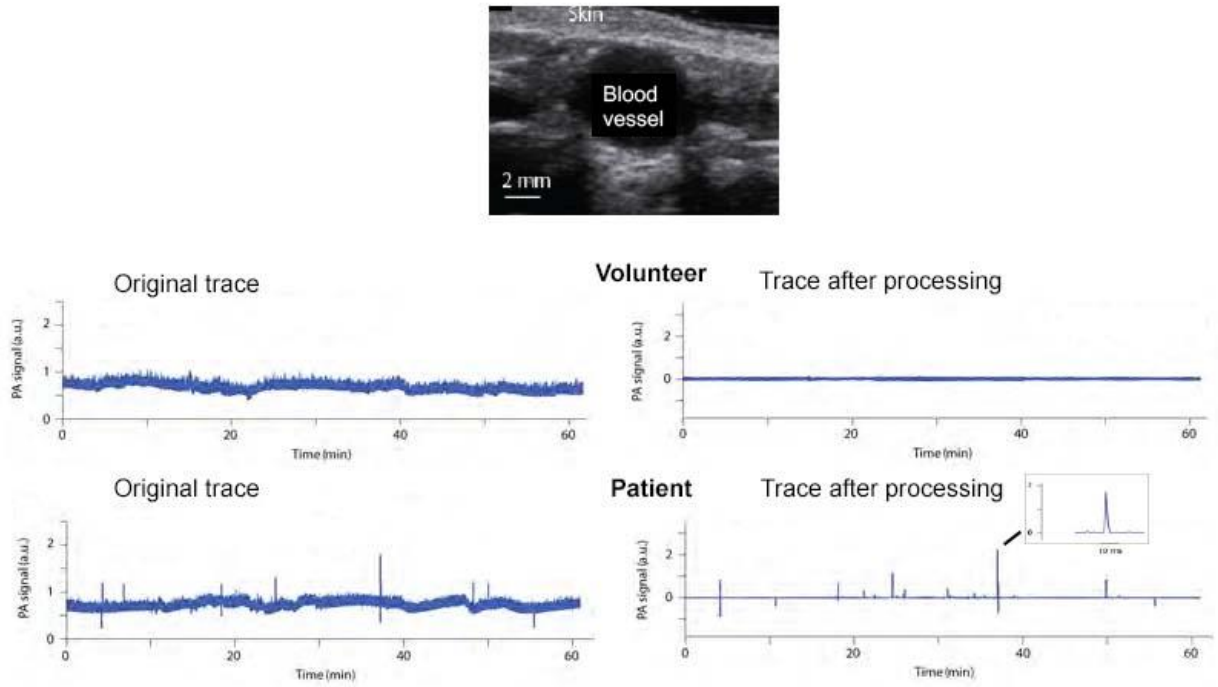

Figure 3. In vivo The Cytophone's detection of CTCs in healthy volunteers and patients. A typical ultrasound image of a vein (top) in the dorsum of the hand and the representative examples of PA traces from a healthy volunteer and a patient with melanoma before and after signal processing.

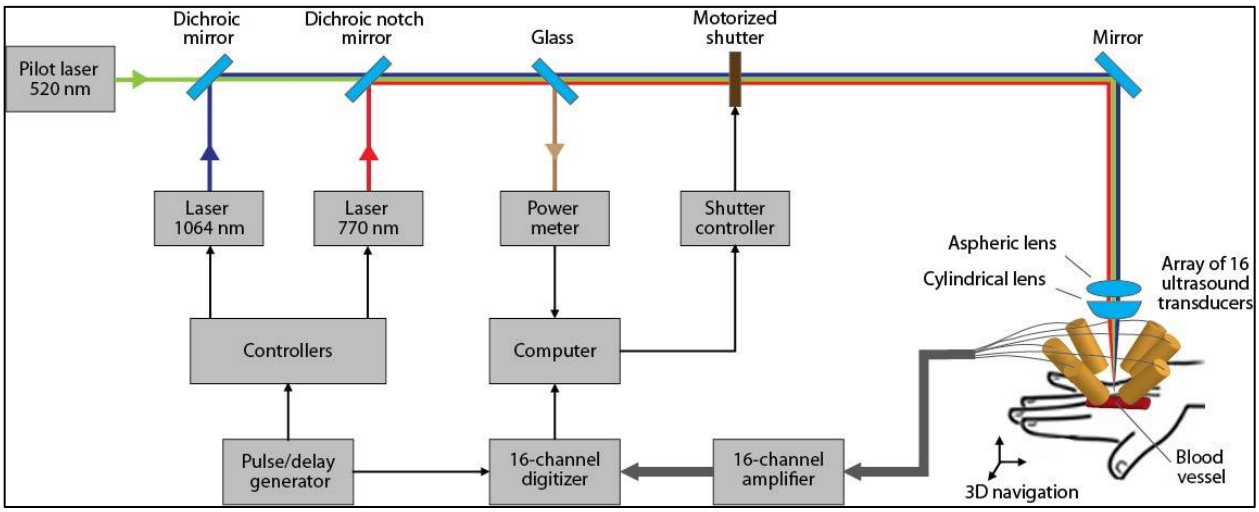

Figure 4. Optical schematic of PAFC -based The Cytophone device.

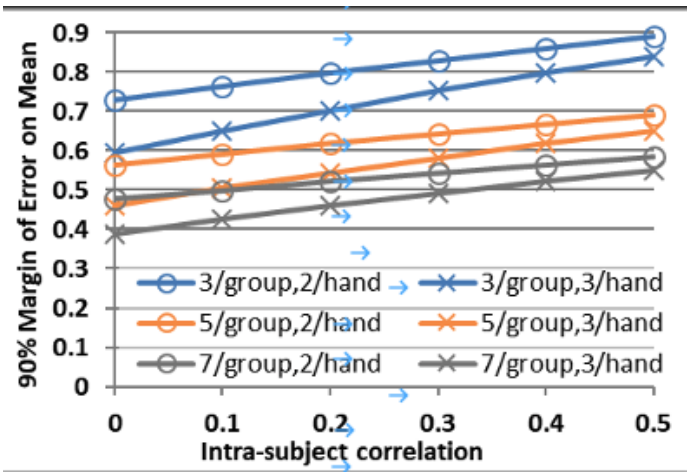

Figure 5. 90% Margin of MEM versus intra-subject correlation for exemplar skin-pigmentation group sizes of 3, 5, and 7 per group when the total sample size is 30. The 90% MEMs (in SD units) are for the group means of measurements taken from the hands of study subjects. They are calculated using the method of Donner under the assumptions (1) that PA contrasts will be determined on exactly 2 vessels/hand ("O" symbols) or exactly 3 vessels/hand ("X" symbols) and (2) that the denominator degrees of freedom will be 12 based on all three skin-pigmentation groups being represented among the 30 study subjects.

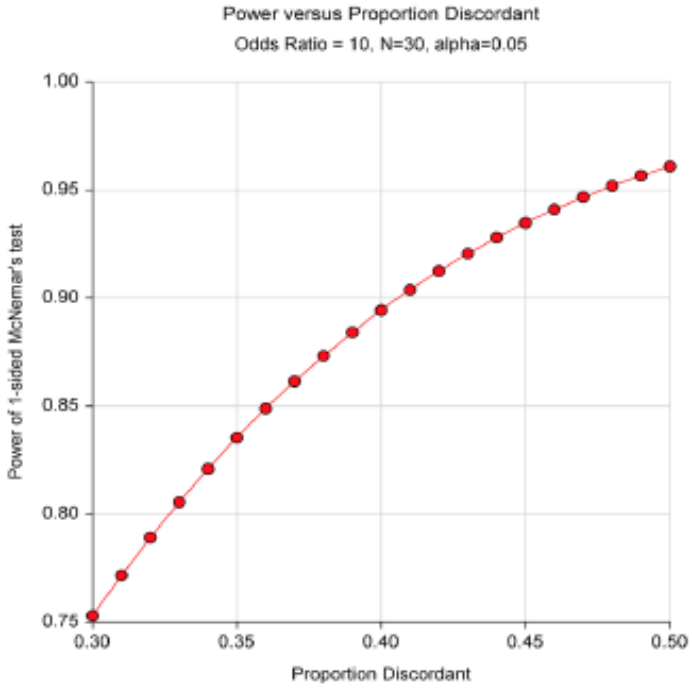

Figure 6. Power versus proportion of discordant pairs of assay results among N=30 total samples from melanoma subjects when the odds ratio among discordant pairs is 10-fold in favor of the Cytophone showing the positive detection result. Power is power of the one-sided McNemar's test at 5% alpha.