Science Translational Medicine
AAAS

stm.sciencemag.org/cgi/content/full/11/496/eaat5857/DC1

Supplementary Materials for

In vivo liquid biopsy using Cytophone platform for photoacoustic detection of circulating tumor cells in patients with melanoma

Ekaterina I. Galanzha, Yulian A. Menyaev, Aayire C. Yadem, Mustafa Sarimollaoglu, Mazen A. Juratli, Dmitry A. Nedosekin, Stephen R. Foster, Azemat Jamshidi-Parsian, Eric R. Siegel, Issam Makhoul, Laura F. Hutchins, James Y. Suen, Vladimir P. Zharov*

*Corresponding author. Email: zharovvladimirp@uams.edu

Published 12 June 2019, *Sci. Transl. Med.* **11**, eaat5857 (2019)
DOI: 10.1126/scitranslmed.aat5857

This PDF file includes:

Supplementary Materials:

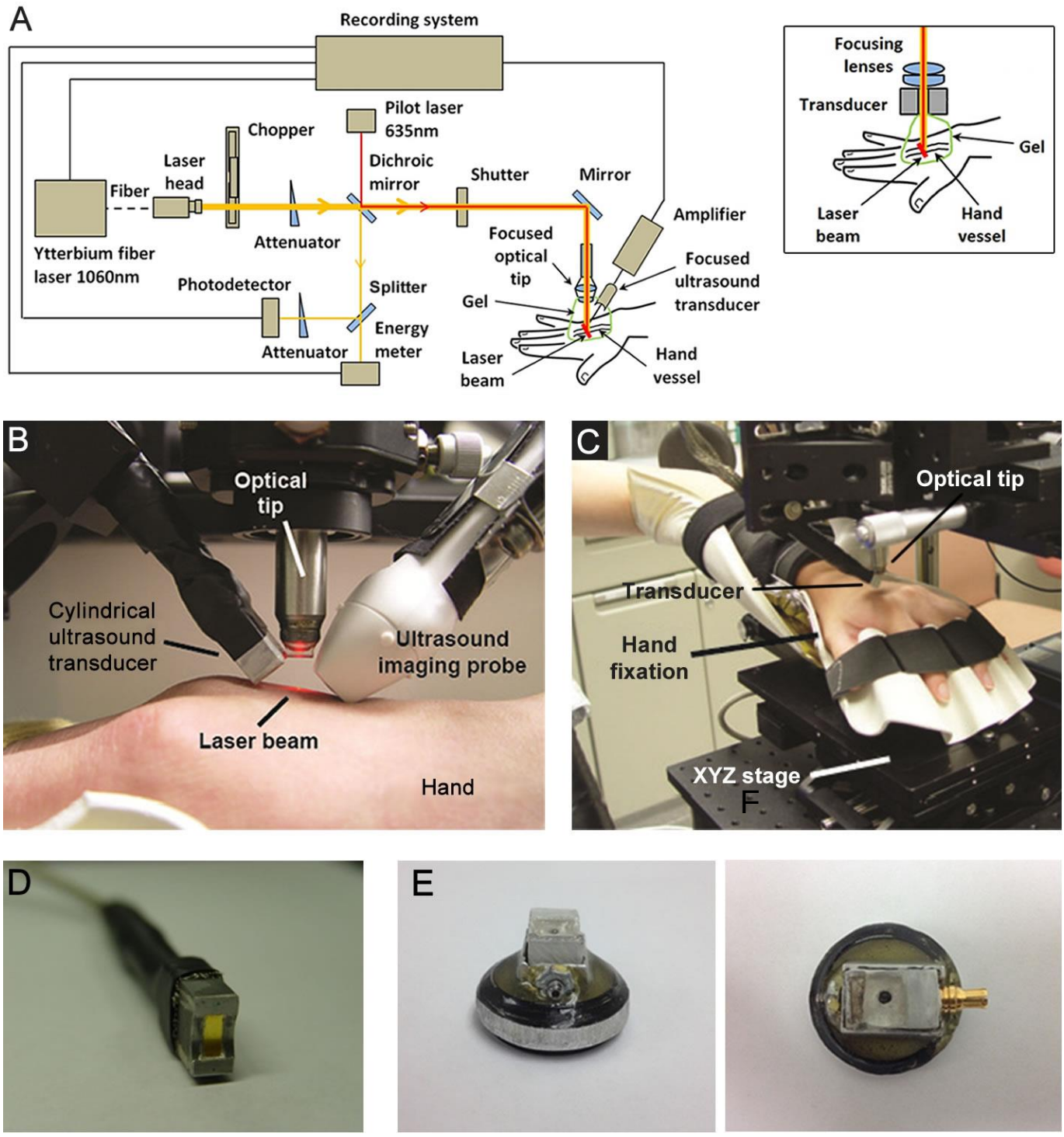

Fig. S1. In vivo acoustic resolution PAFC clinical prototype. (**A)** The optical scheme of PAFC using a high pulse repetition rate laser and a focused cylindrical ultrasound transducer without a central hole (left) and with a central hole (right). (**B**) A photograph of a PA probe consisting of an optical tip with a cylindrical lens for focusing the linear laser beam to the patient's skin and a cylindrical focused transducer (without the hole) located at 30 degrees from the optical tip. The image also shows a standard ultrasound imaging probe, which was used for periodic visualization and measurement of the vein parameters. (**C)** Gentle fixation of the examined hand with a customized holder. (**D** and **E)** The focused cylindrical transducer without a hole (**D**) and with a hole **(E)** at different views.

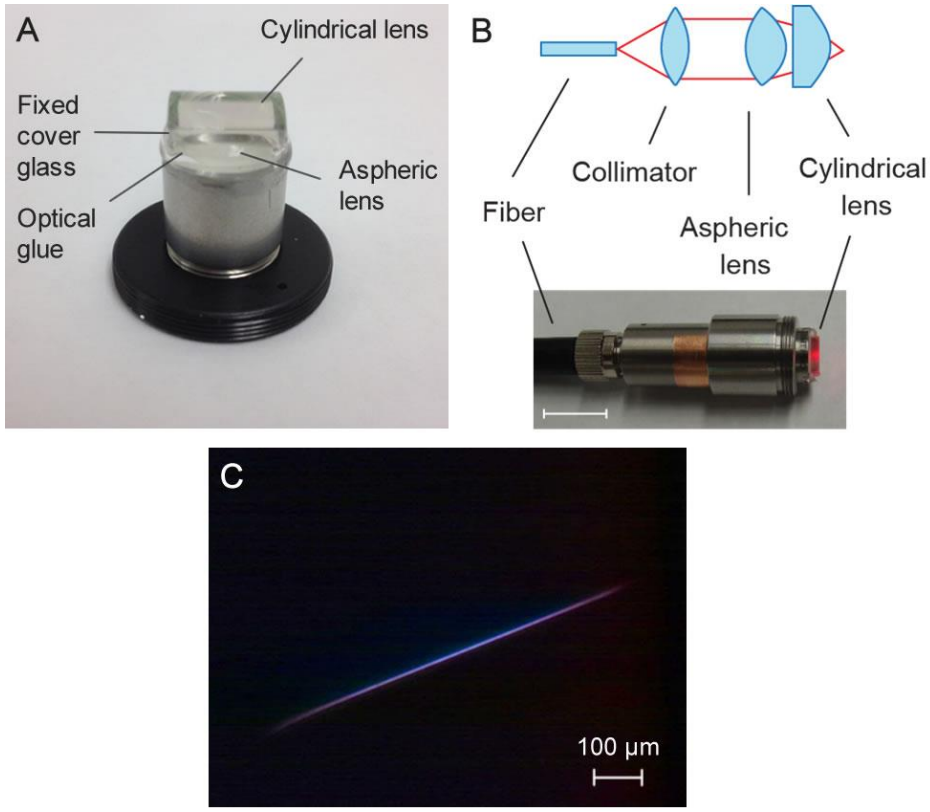

Fig. S2. Optical schematics for delivery of laser radiation to skin. (**A)** The optical tip consisting of an aspheric and a cylindrical lens. (**B)** A schematic of the optical tip (top) and its photo (bottom). (**C)** An image of the linear laser beam with a size of 10 µm x 890 µm. By changing the lens parameters, the size of linear laser beam can be varied from 8 µm x 100 µm to 20 µm x 1850 µm.

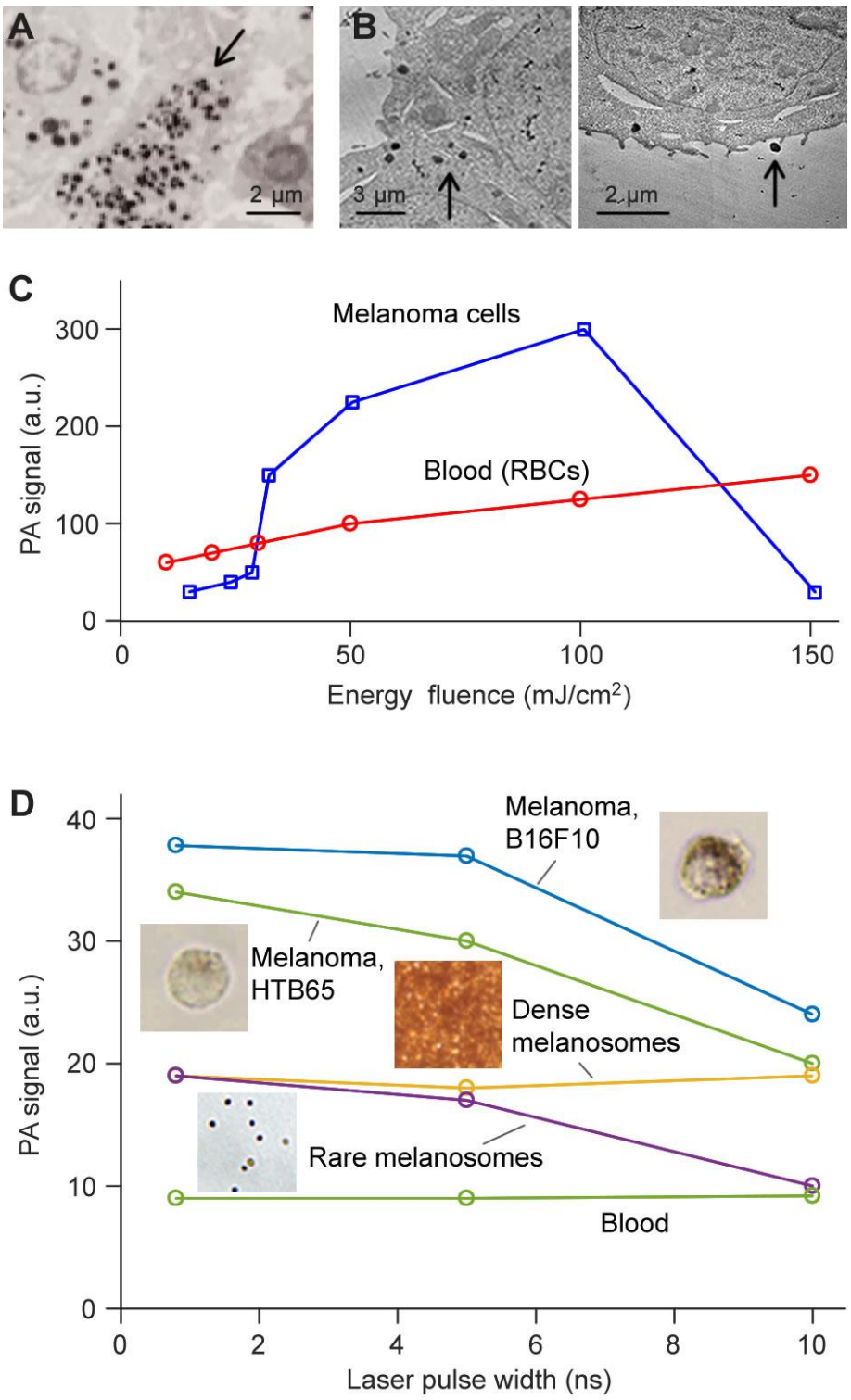

Fig. S3. Optimization of laser parameters in the PAFC setup for detection of CTCs. (**A** and **B)** TEM images of melanoma cell fragments: B16F10 (**A)** and HTB65 **(B)** demonstrating melanin granules (arrows) with sizes in the range of 300-600 nm (left) and 100-250 nm (right) likely as large exosomes. (**C)** The dependence of PA signal amplitudes from melanoma cells and blood on the laser energy fluence. (**D)** The dependence of PA signal amplitudes on laser pulse width for different targets, from cells to ~0.9 µm melanosomes. Laser parameters: wavelength, 1060 nm; pulse repetition rate, 10 kHz; laser beam, circular with diameter of 1 mm; pulse widths, 800 ps, 5 ns, and 10 ns. Ultrasound cylindrical transducer parameters: frequency, 0-32 MHz; focal distance, 6 mm; lateral resolution, 65 ± 6 µm. The samples were put on microscope slides with a thickness of 120 µm.

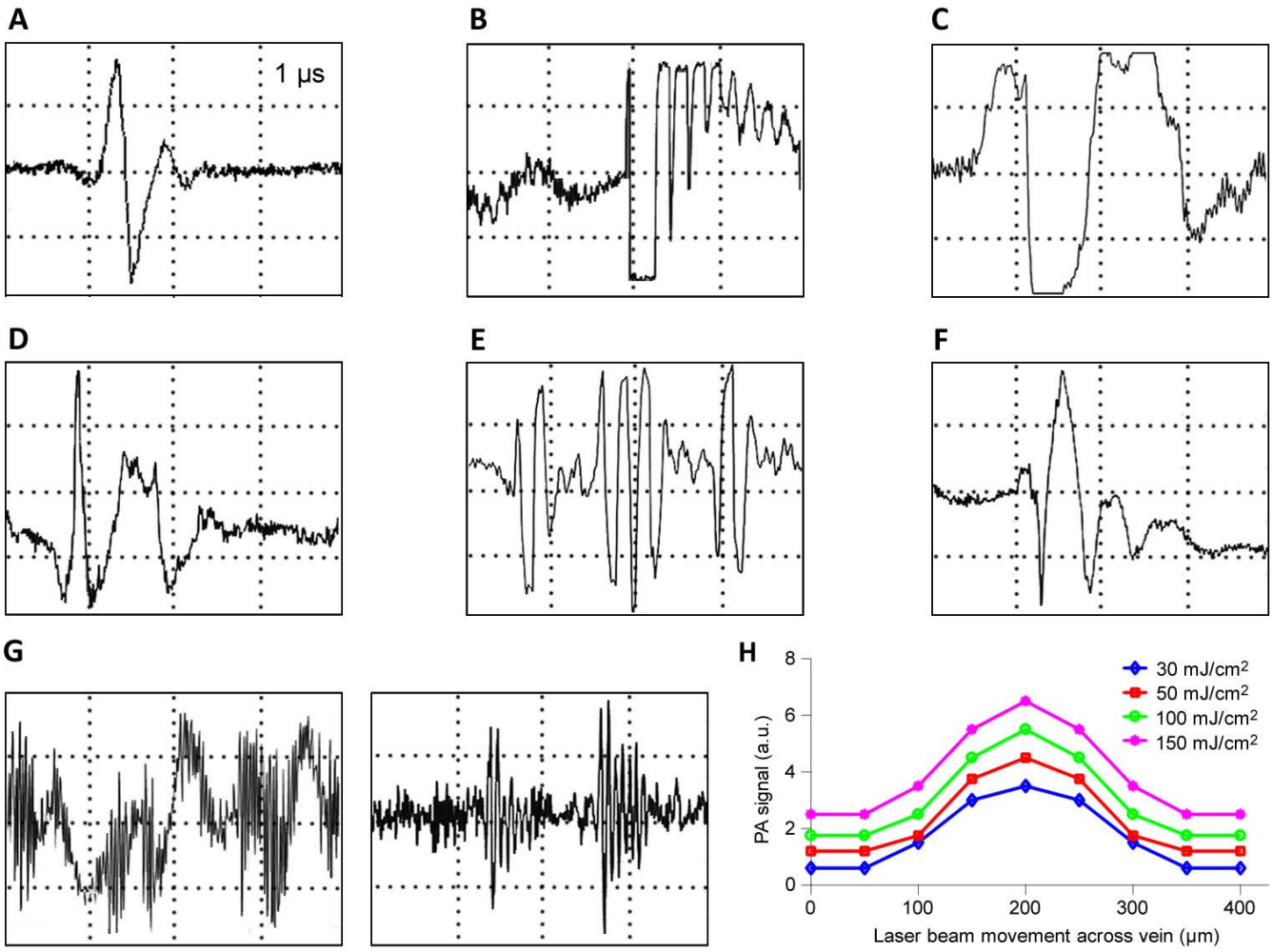

Fig. S4. Typical examples of PA signal amplitude and shape from vessels and artifacts. (A) PA signal from blood vessel (background). **(B)** Influence of electrical shutter. **(C)** Impact of quick door closing. (**D)** The source of signal is a hair during slight beam movement. **(E**) Influence of medical ultrasound system. **(F)** Moving bubble in ultrasound gel. (**G)** Artifacts generated by a high-power unit (generator) in the same building. The unit of the horizontal axis is 1 µs. (**H)** The dependence of PA signals on the position of the linear laser beam (8 µm x 400 µm) across a 450 µm mouse leg vein using the focused cylindrical transducer with the following parameters: focal distance, 4 mm; lateral resolution, 65 µm.

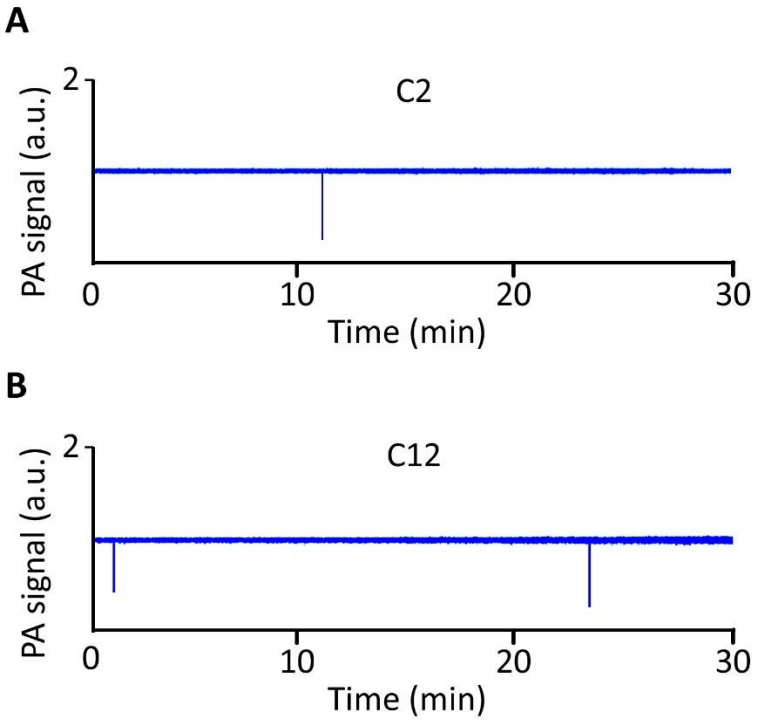

Fig. S5. Examples of small CBCs observed in a few healthy volunteers. (A, B) PA traces from two healthy volunteers showing one **(A)** and two **(B)** negative PA contrast peaks.

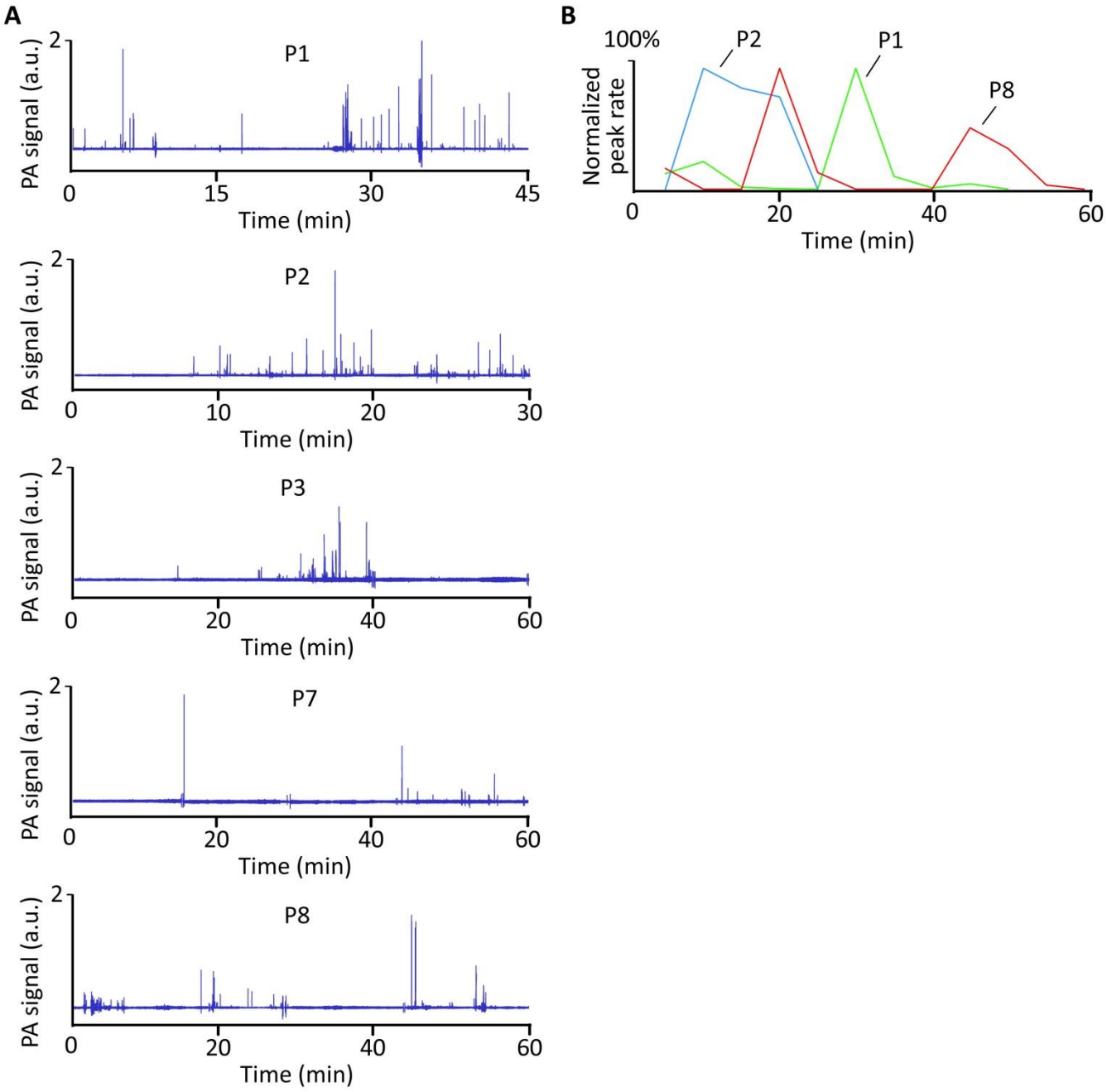

Fig. S6. PA traces from hand veins of patients with melanoma and PA signal rates. (A) Sample PA traces from different patients with melanoma before subtractions of the artifacts. **(B)** PA peak rates (number of peaks per 5 minutes) for selected patients.

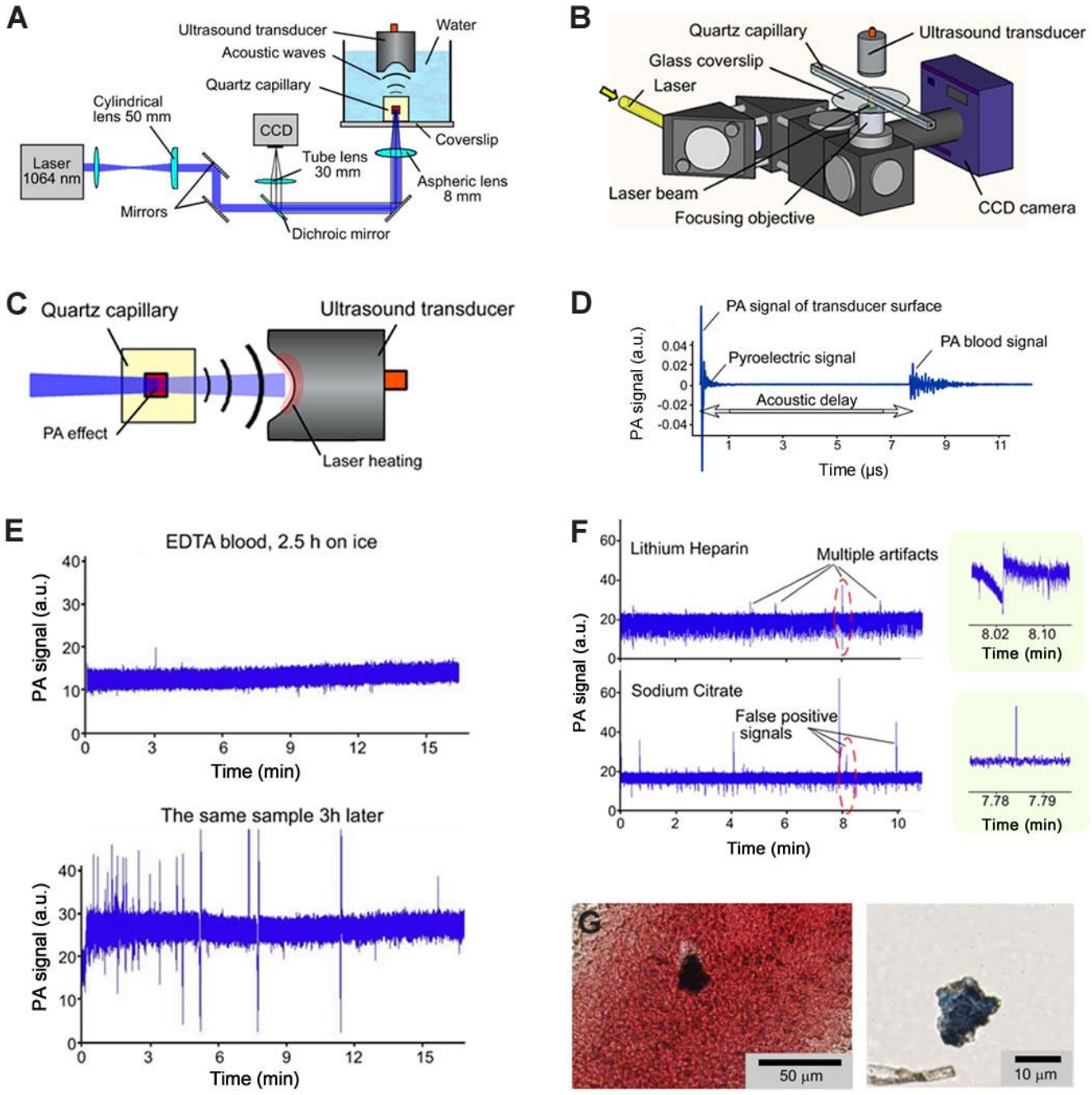

Fig. S7. In vitro PA testing of blood samples from healthy volunteers and patients with melanoma. The second optical schematic of in vitro PAFC **(A)** and the spatial location of its main module **(B).** (**C)** A schematic of the acoustic detection system. **(D)** A typical PA signal from the blood (right) coming with a few μs of time delay to the transducer compared to the artifact (left) induced by direct laser irradiation of the transducer surface. **(E)** The PA trace for an EDTA-stabilized blood sample kept on ice for 2.5 h after the sample was collected **(top)** and 3 h after removal from ice (**bottom)**. **(F)** Typical PA traces of blood samples from healthy volunteers using tubes with citrate **(top**) and heparin **(bottom)** as anticoagulants. Laser parameters: wavelength, 1064 nm; pulse width, 10 ns; pulse rate, 2 kHz; energy fluence, 500 mJ/cm^2. (**G**) Vacutainer cap-related artifacts suspected of causing false positive PA peaks. Left: an artifact found in a small volume of healthy volunteer blood (10 μl) collected after observing high-amplitude PA signals. Right panel shows the same artifact after blood cells were washed out.

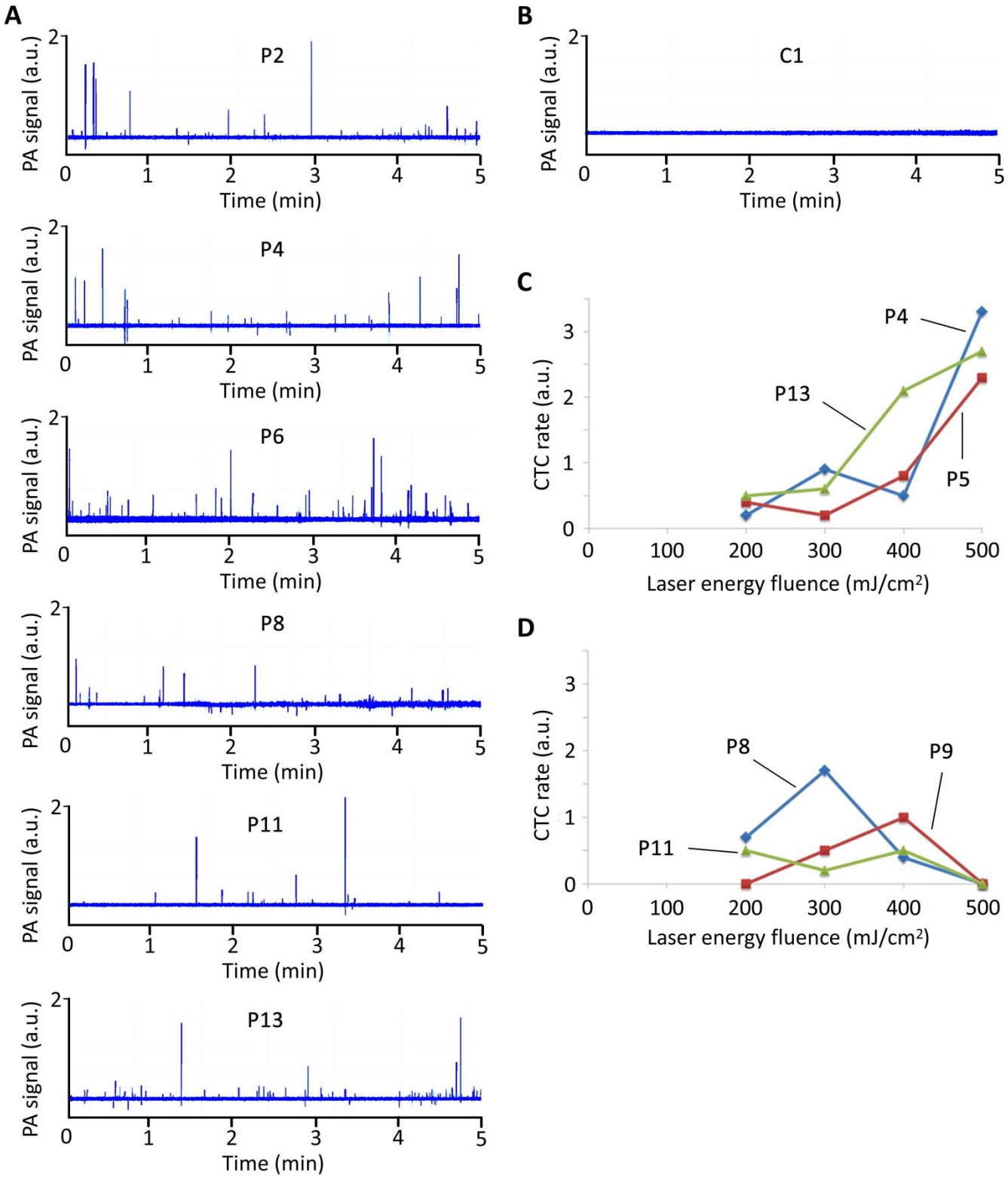

Fig. S8. Examples of PA traces in blood samples from a healthy volunteer and six patients with melanoma in vitro. (**A)** Different patients with melanoma. **(B)** A healthy control individual. (**C** and **D)** The nonlinear dependence of PA signal rates without **(C**) and with **(D)** CTC destruction (CTC theranostics). The blood samples were collected using tubes with EDTA anticoagulant. The traces are presented excluding the artifacts as described in the main text.

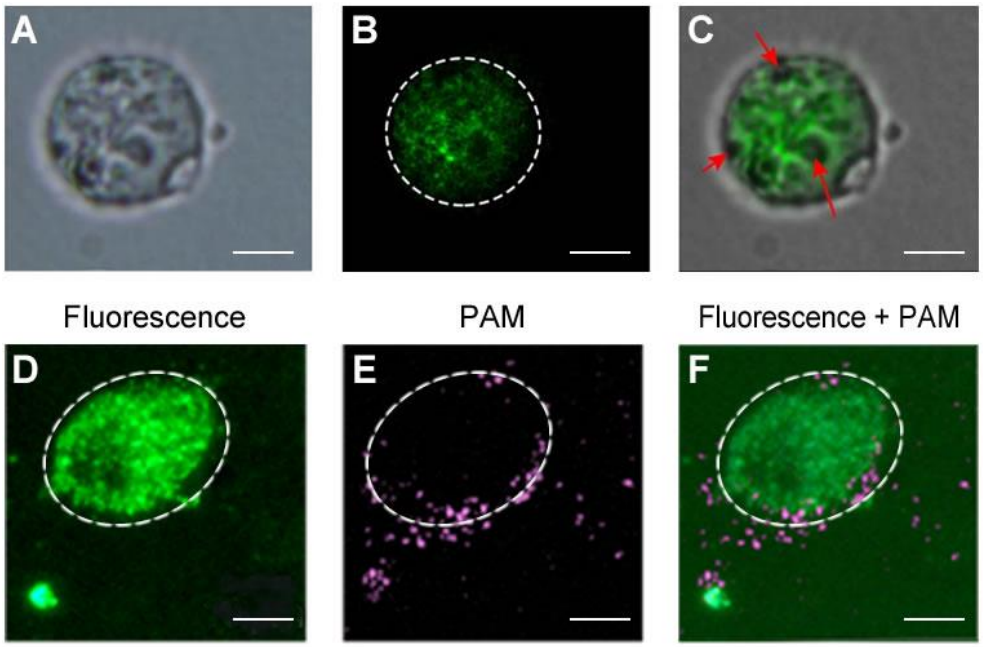

Fig. S9. Melanin-induced quenching of fluorescence in melanoma CTCs. Transmission **(A),** fluorescence **(B),** and combined **(C)** transmission-fluorescence images of a single melanoma cell (B16F10) with genetically induced Dendra2 fluorescence protein. The arrows in C indicate high melanin content resulting in decreased fluorescence intensity. **(D**) Fluorescence image of B16F10 cell with genetically induced green fluorescence protein (GFP). (**E)** PA image of the same cell showing cellular melanin distribution. **(F)** Combined fluorescent-PA image of the same cell indicating quenching effect in zones with high melanin content, such as near the cell membrane. All scale bars are 10 μm.

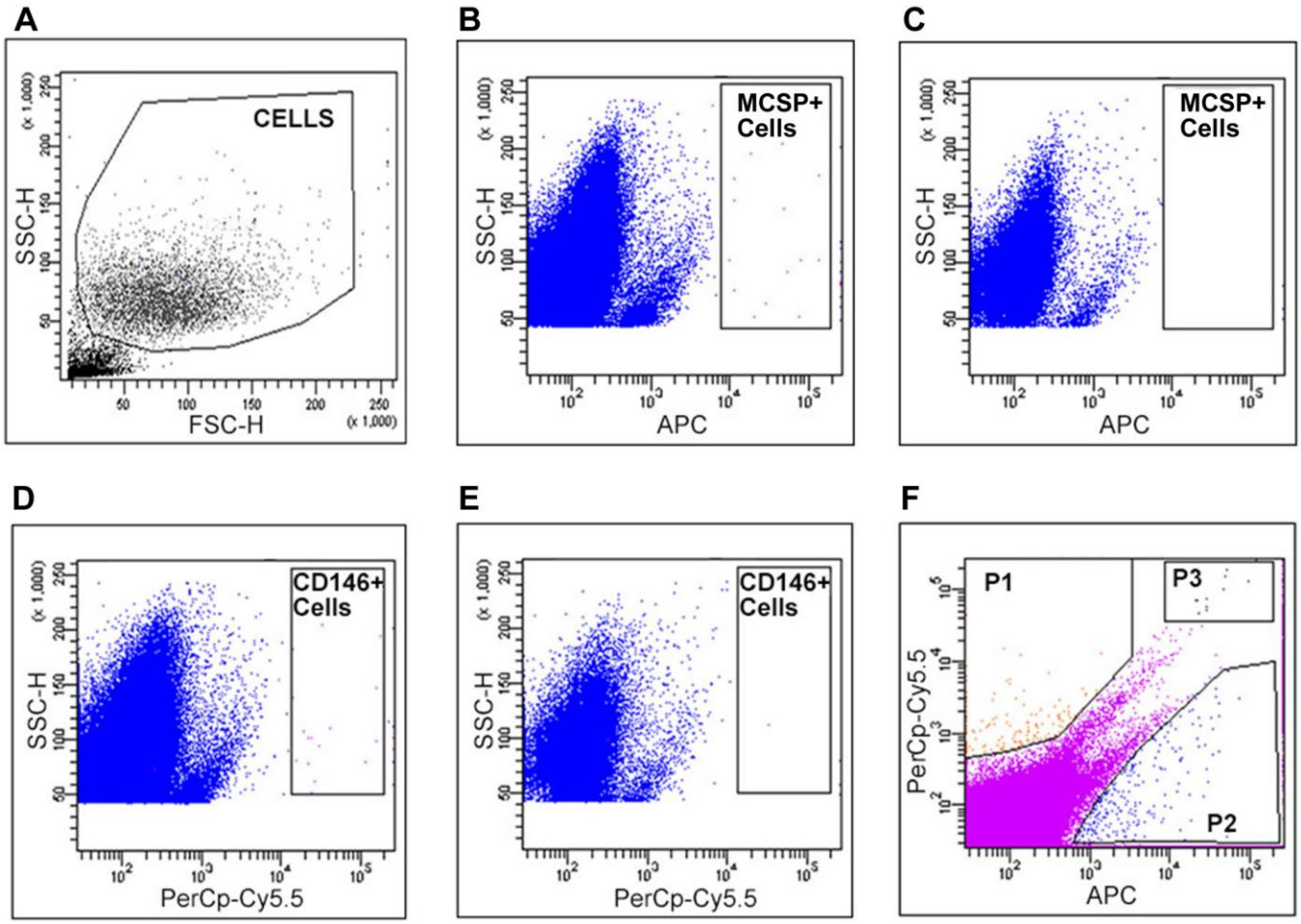

Fig. S10. In vitro conventional flow cytometry of blood samples from a patient with melanoma. (**A)** The gating strategy for identifying mononuclear cells. (**B** and **C)** The data for samples with MCSP+ CTCs **(B)** and without MCSP+ CTCs **(C)**. (**D** and **E)** The data for samples with CD146+ CTCs **(D)** and without CD146+ CTCs **(E)**. (**F)** The data for a sample with MCSP+/CD146+ CTCs: P1 – CD146+ CTCs, P2 – MCSP+ CTCs, and P3 – MCSP+/CD146+ CTCs.

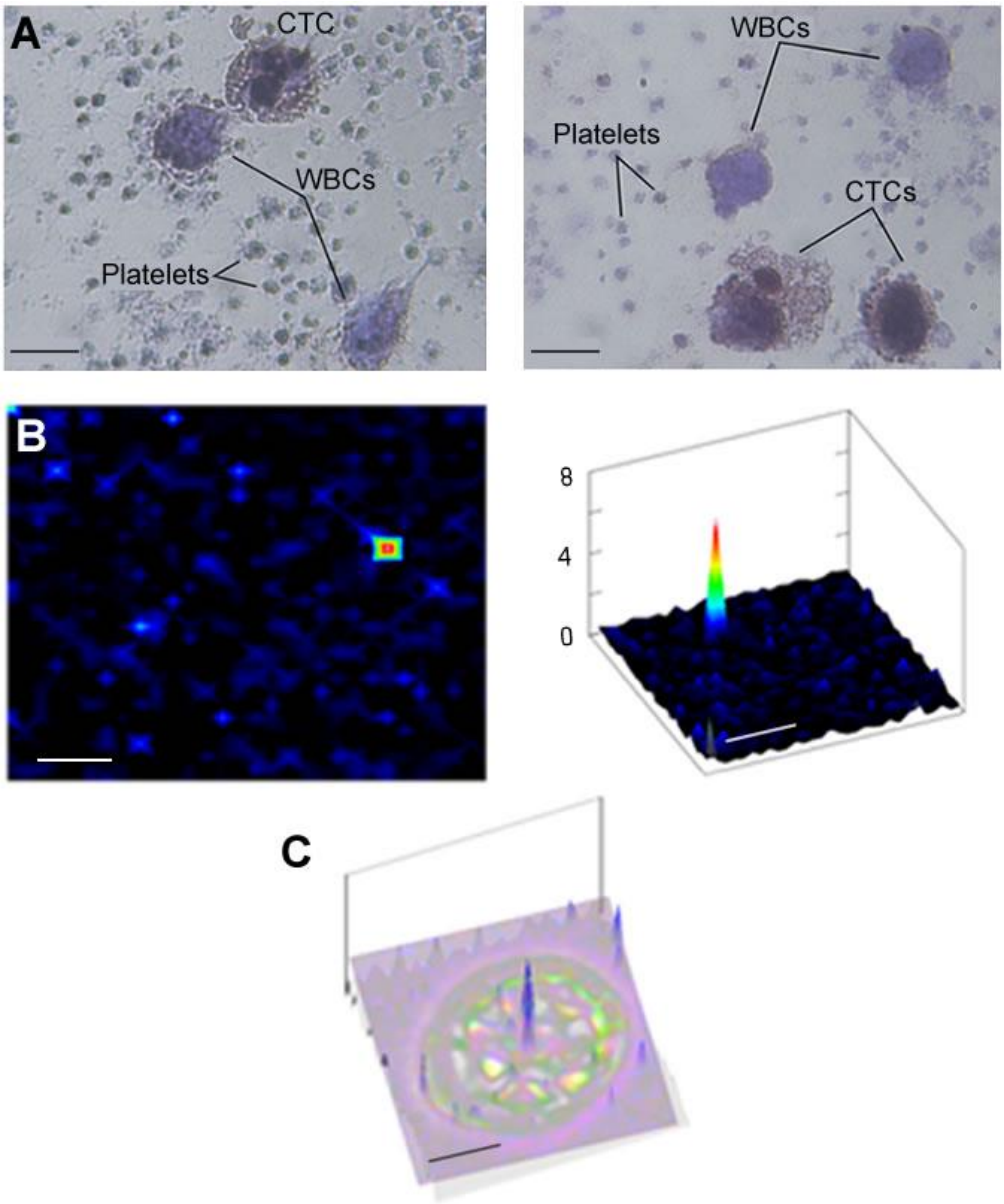

Fig. S11. Melanoma cell detection in patients' blood samples after magnetic enrichment in vitro. (**A**) Immunocytochemical staining procedure for melanoma cells after magnetic separation by using the HiDef Detection HRP Polymer System and melanoma antibody cocktail containing HMB-45, MART-1, and tyrosinase. (**B**) Photothermal (PT) imaging of enriched samples indicating the presence of melanoma cells with magnetic beads. This analysis was performed by scanning the focused 2 μm laser beam across the sample (scanning PT microscopy). The results of this analysis were presented as 2-D (left) and 3-D (right) simulation. (**C**) Magnetic microbeads on the surface of a melanoma cell. The scale bars in panels A through C represent 10 μm.

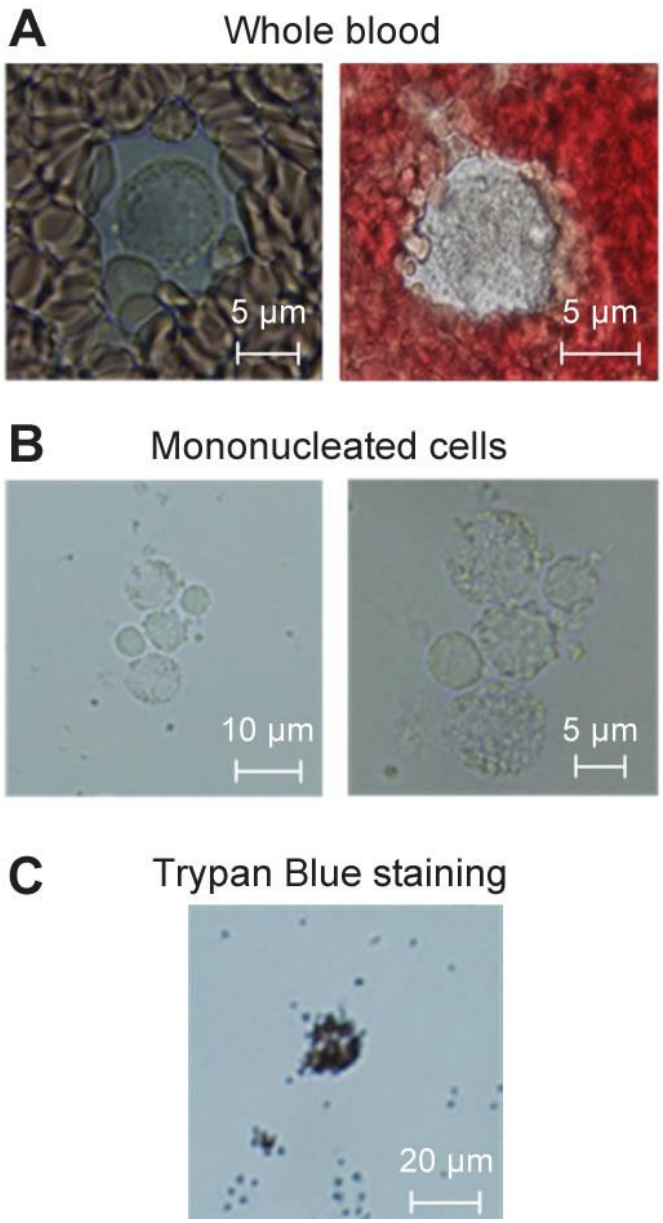

Fig. S12. Visualization of cells using staining procedures. (A) Whole blood images taken at 40x (left) and 100x (right) magnification, showing a leukocyte within peripheral blood. (**B**) Leukocytes remaining after isolation of the mononuclear layer. (**C**) Staining of melanoma cell with Trypan Blue. The viability of melanoma cells was 56 ± 6.6%, while leukocyte viability was 89 ±6.9%.

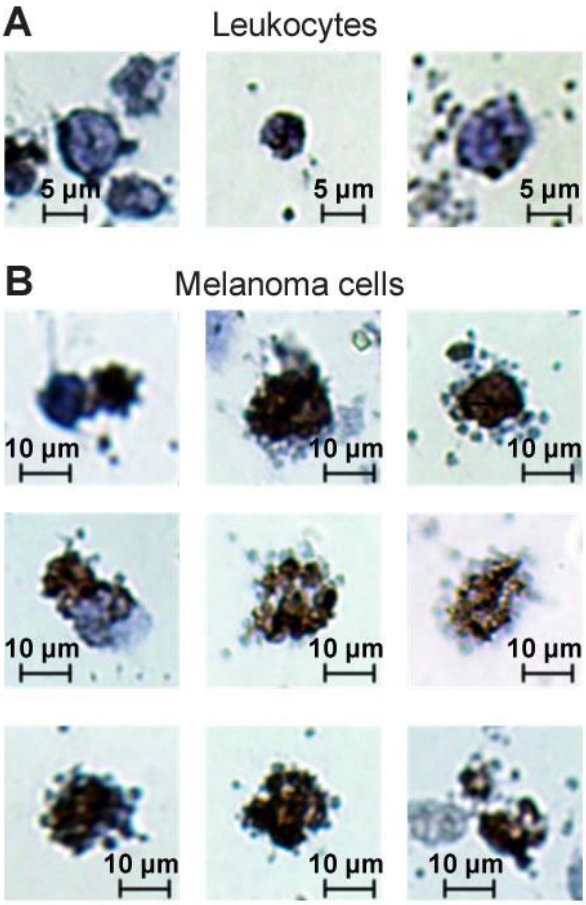

Fig. S13. Immunocytochemical analysis of blood samples from patients with melanoma. (**A and B**) Identification of leukocytes **(A)** and melanoma cells **(B)**, using hemalum blue staining and anti-melanoma brown stain, respectively. Some melanoma cells are in a necrotic state.

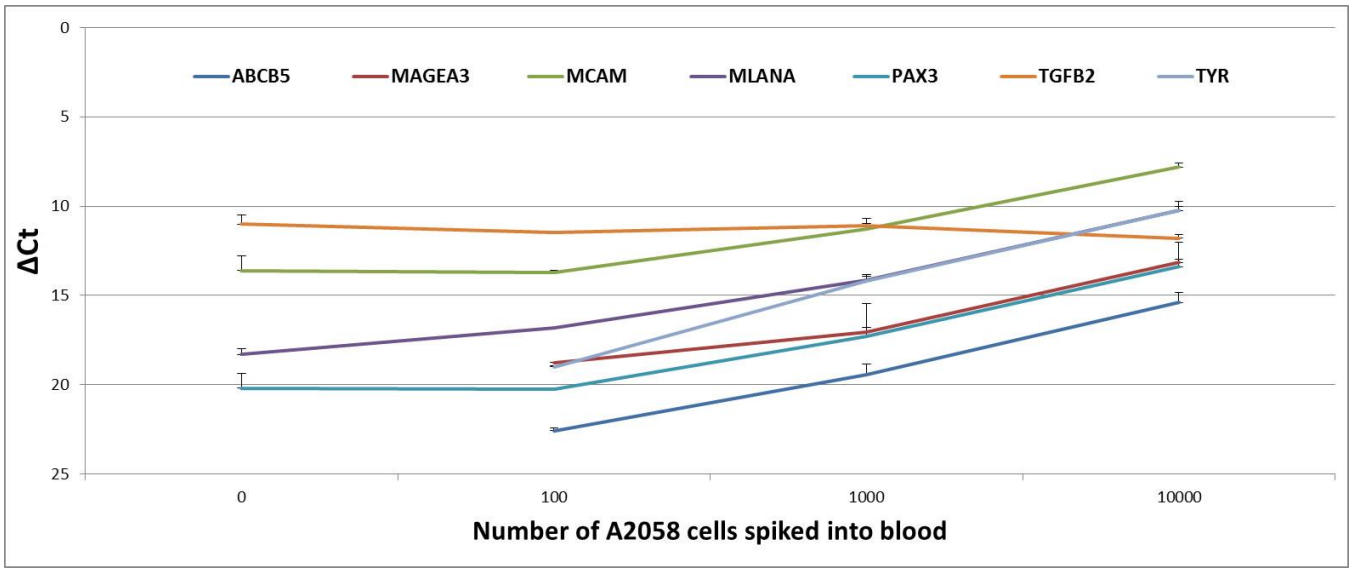

Fig. S14. Sensitivity of qRT-PCR for detecting melanoma markers in healthy blood sample spiked with A2058 human melanoma cells. ΔCt in vertical axis represents the difference in Ct values between melanoma target genes and those of housekeeping *GAPDH* gene.

Table S1. Patient parameters.

	Subject #	Sex	Race	Melanoma stage	Diameter, min - max (mm)	Depth (mm)	Flow velocity (cm/s)
Patients	P1	F	W	IV	1.7 - 1.9	1.8	3.46
	P2	M	W	IV	1.3 - 1.5	1.2	5.22
	P3	M	W	IV	2.5 - 2.5	1.8	4.70
	P4	M	W	IIC	2.3 - 2.3	1.9	4.43
	P5	M	W	IV	1.3 - 1.3	1.2	3.13
	P6	M	W	III	2.2 - 2.2	1.1	3.62
	P7	M	W	IV	2.3 - 2.6	1.5	3.95
	P8	M	W	III	2.8 - 3.2	1.5	4.12
	P9	F	W	IV	2.3 - 2.4	1.3	4.12
	P10	F	W	IV	2.2 - 2.2	1.1	3.62
	P11	F	W	III	1.3 - 1.4	1.4	3.13
	P12	F	W	IV	1.3 - 1.3	1.5	4.12
	P13	F	W	III	2.3 - 2.4	1.6	4.12
	P14	M	W	IV	2.0 - 2.1	1.2	4.28
	P15	M	W	IV	1.7 - 1.9	1.8	3.13
	P16	F	W	IV	~1.9	N/A	N/A
	P17	F	W	IV	N/A	N/A	N/A
	P18	M	W	IV	~2.1	N/A	N/A
	P19	M	W	III	1.7 – 1.8	1.0	4.00
	P20	F	W	IV	0.9 – 1.1	0.9	5.43
	P21	M	W	III	1.6 – 1.6	1.4	4.12
	P22	F	W	III	1.7 – 1.8	1.4	4.61
	P23	M	W	III	1.5 – 1.6	1.3	3.62
	P24	F	W	IV	1.6 – 1.6	4.2	6.00
	P25	M	W	IIA	0.9 – 1.3	1.0	3.00
	P26	M	W	IV	1.4 – 1.5	1.1	3.95
	P27	M	W	II	1.8 – 2.1	2.1	4.75
	P28	F	W	IV	1.2 – 1.3	0.8	4.12
Healthy volunteers	C1	M	W		2.0 - 2.0	2.0	4.00
	C2	M	W		2.1 - 3.1	1.1	4.96
	C3	M	W		1.3 - 1.6	1.1	4.70
	C4	M	W		1.4 - 2.2	1.4	5.48
	C5	M	W		1.4 - 1.9	2.1	6.09
	C6	F	W		2.0 - 2.0	2.0	4.00
	C7	F	W		0.9 - 0.9	1.6	2.47
	C8	M	AA		1.3 - 1.9	1.5	3.46
	C9	F	W		1.3 - 2.3	1.6	4.61
	C10	F	AA		1.3 - 1.3	1.0	4.77
	C11	F	W		2.9 - 2.9	1.9	4.70
	C12	M	W		4.3 - 4.6	1.4	7.50
	C13	M	AA		1.7 - 1.7	1.9	4.12
	C14	M	W		N/A	N/A	N/A
	C15	F	AA		N/A	N/A	N/A
	C16	M	AA		3.6 – 3.9	1.6	5.60
	C17	M	W		N/A	N/A	N/A
	C18	F	W		N/A	N/A	N/A
	C19	F	W		N/A	N/A	N/A

M: Male, F: Female, W: White, AA: African American, N/A: Not available
Diameter, min-max means the size of noncircular vessels in two orthogonal directions.

Table S2. Detection of CTCs in melanoma patients with PAFC in vitro.

CTCs	in vitro PAFC			
Subject #	**Blood volume†**	**Number detected**	**CTCs per mL estimate±SE‡**	**Patient Result§**
P1	1.33	23	17.29 ± 3.61	Yes
P2	1.61	13	8.07 ± 2.24	Yes
P3	1.61	2	1.24 ± 0.88	No
P4	1.65	1	0.61 ± 0.61	No
P5	1.56	0	0.00 ± 0.00	No
P6	1.63	0	0.00 ± 0.00	No
P7	1.63	1	0.61 ± 0.61	No
P8	1.63	1	0.61 ± 0.61	No
P9	1.69	0	0.00 ± 0.00	No
P10	1.56	0	0.00 ± 0.00	No
P11	1.64	0	0.00 ± 0.00	No
P12	1.65	0	0.00 ± 0.00	No
P13	1.62	0	0.00 ± 0.00	No
P14	1.65	1	0.61 ± 0.61	No
P15	1.68	0	0.00 ± 0.00	No
P16	N/A	N/A	N/A	N/A
P17	N/A	N/A	N/A	N/A
P18	N/A	N/A	N/A	N/A
P19	3.79	10	2.64 ± 0.83	Yes
P20	3.99	19	4.76 ± 1.09	Yes
P21	4.81	2	0.42 ± 0.29	No
P22	4.86	8	1.65 ± 0.58	No
P23	4.76	7	1.47 ± 0.56	No
P24	4.94	3	0.61 ± 0.35	No
P25	4.81	1	0.21 ± 0.21	No
P26	4.81	4	0.83 ± 0.42	No
P27	4.69	1	0.21 ± 0.21	No
P28	4.64	1	0.22 ± 0.22	No
For P1-P15: CTCs per ml in vivo versus in vitro: Spearman correlation = +0.570 (P=0.0266)				
CTC patient result in vivo versus in vitro: Concordance=3/15 (20%); Cohen's κ ±SE = 0.02 ± 0.13				
For P19-P28: CTCs per ml in vivo versus in vitro: Spearman correlation = +0.527 (P=0.1172)				
CTC patient result in vivo versus in vitro: Concordance=2/10 (20%); Cohen's κ ±SE = 0.00 ±0.16				

†: Blood volume is in ml.

‡: ±Standard Error.

§: Patient result was calculated as Yes if the patient's CTC rate (in CTCs/ml) was elevated above the false-positive CTC rate by >2 standard errors of the difference. False-positive CTC rates ±SEs were 0.0040±0.0008 CTCs/ml in vivo and 0.465±0.147 CTCs/ml in vitro.

Table S3. Detection of CBCs in melanoma patients with PAFC in vitro.

CBCs	in vitro PAFC			
Subject #	**Blood volume†**	**Number detected**	**CBCs per mL estimate±SE‡**	**Patient Result§**
P1	1.33	12	9.02 ± 2.60	Yes
P2	1.61	5	3.11 ± 1.39	No
P3	1.61	30	18.63 ± 3.40	Yes
P4	1.65	0	0.00 ± 0.00	No
P5	1.56	0	0.00 ± 0.00	No
P6	1.63	0	0.00 ± 0.00	No
P7	1.63	0	0.00 ± 0.00	No
P8	1.63	0	0.00 ± 0.00	No
P9	1.69	0	0.00 ± 0.00	No
P10	1.56	0	0.00 ± 0.00	No
P11	1.64	0	0.00 ± 0.00	No
P12	1.65	0	0.00 ± 0.00	No
P13	1.62	1	0.62 ± 0.62	No
P14	1.65	0	0.00 ± 0.00	No
P15	1.68	0	0.00 ± 0.00	No
P16	N/A	N/A	N/A	N/A
P17	N/A	N/A	N/A	N/A
P18	N/A	N/A	N/A	N/A
P19	3.79	0	0.00 ± 0.00	No
P20	3.99	0	0.00 ± 0.00	No
P21	4.81	0	0.00 ± 0.00	No
P22	4.86	2	0.41 ± 0.29	No
P23	4.76	2	0.42 ± 0.30	No
P24	4.94	0	0.00 ± 0.00	No
P25	4.81	0	0.00 ± 0.00	No
P26	4.81	0	0.00 ± 0.00	No
P27	4.69	1	0.21 ± 0.21	No
P28	4.64	0	0.00 ± 0.00	No
For P1-P15: CBCs per ml in vivo versus in vitro: Spearman correlation = +0.024 (*P*=0.93) CBC patient result in vivo versus in vitro: Concordance=12/15 (80%); Cohen's κ ±SE = –0.10 ±0.57 For P19-P28: CTCs per ml in vivo versus in vitro: Spearman correlation = +0.450 (*P*=0.1921) CTC patient result in vivo versus in vitro: Concordance=10/10 (100%)				

†: Blood volume is in ml.

‡: ±Standard Error.

§: Patient result was calculated as Yes if the patient's CBC rate (in CBCs/ml) was elevated above the false-positive CBC rate by >2 standard errors of the difference. False-positive CBC rates ±SEs were 0.000±0.000 CBCs/ml in vivo and 0.372±0.131 CBCs/ml in vivo.

Table S4. Results of testing the blood samples from patients with melanoma by flow cytometry in vitro.

Subject #	MCSP+/ CD146-/ CD45-	MCSP-/ CD146+/ CD45-	MCSP+/ CD146+/ CD45-
P1	+	+	+
P2	+	-	-
P3	±	±	±
P4	-	-	-
P5	-	+	-
P6	-	-	-
P7	-	+	-
P8	-	-	-

Table S5. Relative gene expression profile of melanoma markers in patients with melanoma. qRT-PCR data showing fold changes in expression of the markers in patient samples normalized to the negative controls. Values higher than 1.5-fold are represented in bold font.

Subject#	ABCB5	MAGEA3	MCAM	MLANA	PAX3	TGFB2	TYR
P1	0.5	N/A	0.5	0.7	**17.2**	0.6	0.8
P2	1.1	N/A	1.1	0.7	**28.4**	0.5	0.8
P3	0.4	N/A	0.6	**2.8**	**5.7**	0.9	**2.3**
P4	0.7	N/A	**2.1**	**3.5**	**5.3**	1.1	**10.2**
P5	N/A	N/A	0.8	**4.2**	**3.3**	1.1	N/A
P6	0.4	N/A	1.1	0.6	0.6	0.7	0.5
P7	**2.3**	N/A	1.1	**9.7**	**5.0**	0.2	**23.3**
P8	0.8	N/A	1.0	0.9	**19.2**	0.3	N/A
P9	1.3	N/A	0.7	0.3	**68.1**	0.8	N/A
P10	N/A	N/A	0.5	0.5	0.7	0.6	N/A
P11	N/A	N/A	1.4	0.3	**18.4**	0.5	N/A
P12	N/A	N/A	1.1	0.7	**2.8**	0.4	**9.7**
P13	0.3	N/A	0.4	0.8	**3.0**	0.4	N/A
P14	0.5	N/A	0.9	0.1	**1.5**	0.2	N/A

Table S6. Comparison of data for selected patients in vivo with the results ex vivo using different in vitro assays. The symbols "+" and "-" mean the confirmed presence and absence of CTCs in the samples, respectively. The symbol "±" indicates disagreements ascribed to the high false positivity rate and low detection limit of most CTC assays in vitro.

Subject #	In vivo PAFC CTCs/mL	In vitro PAFC	In vitro FC	MACS	qRT-PCR	Fluorescent imaging	PTM
P1	1.178	+	+	±	+	+	+
P2	2.033	+	-	-	+	+	+
P3	0.143	±	±	-	+	±	±
P4	0.046	±	-	-	+	±	-
P5	0.000	-	±	±	±	-	-
P6	0.006	-	-	-	-	-	-
P7	0.022	±	±	±	+	±	±
P8	0.075	±	-	-	±	-	-

PAFC – photoacoustic flow cytometry;
FC – flow cytometry (conventional);
MACS – magnetic-activated cell sorting;
qRT-PCR – quantitative reverse transcription-polymerase chain reaction;
PTM – photothermal microscopy.