In vivo blood flow and lymphatic examination for early diagnosis and therapy of tumor metastasis

Ekaterina Galanzha & Vladimir Zharov

Philips Classic Laser & Nanomedicine Laboratories
Winthrop P. Rockefeller Cancer Institute
University of Arkansas for Medical Sciences, Little Rock, AR

Little Rock Capitol Building

"Gone With the Wind": the Old Mill

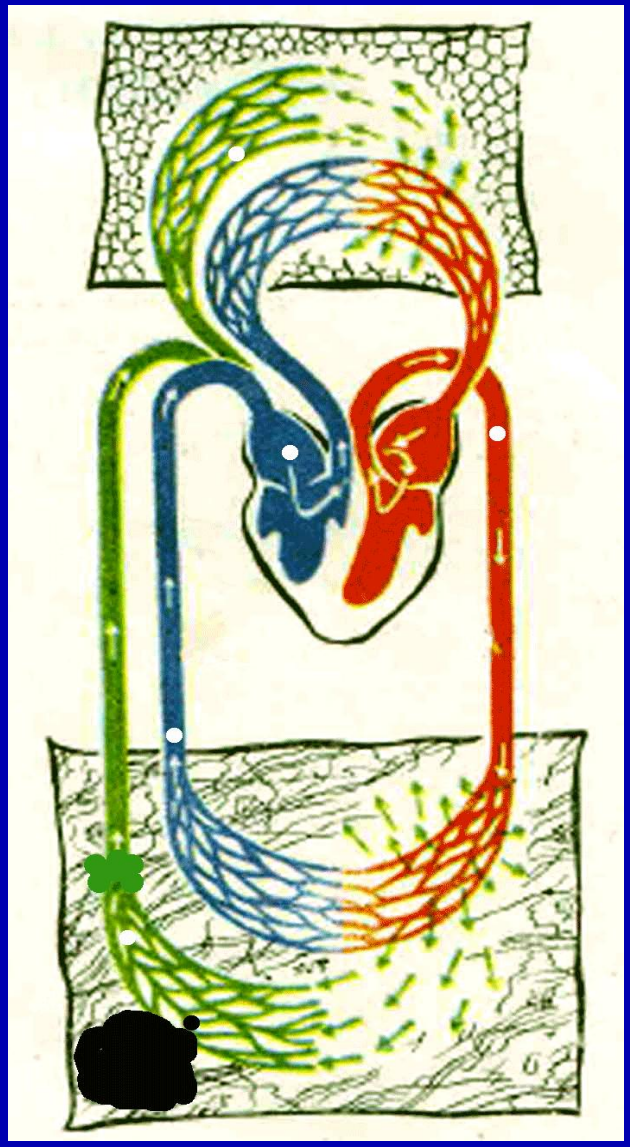
The University of Arkansas for Medical Sciences (UAMS)

The Winthrop P. Rockefeller Cancer Institute

Motivation/Goal

In vivo noninvasive blood and lymph testing for early disease diagnosis and personalized therapy by integration of the principles of flow cytometry, photoacoustics and laser spectroscopy with nanoparticles as high-contrast molecular agents. New testing provides *in vivo* enumeration, enrichment, and eradication of circulating tumor cells directly in the blood circulation

Diagnosis of metastasis

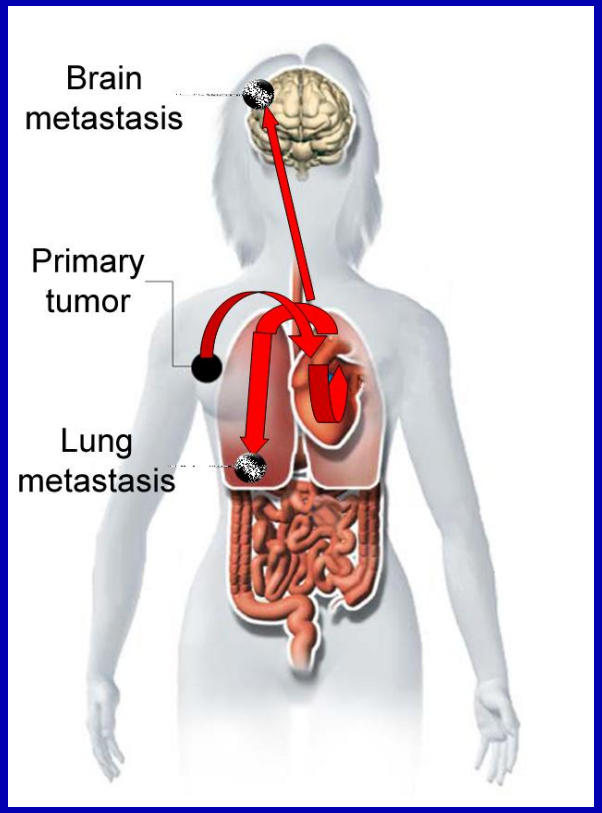


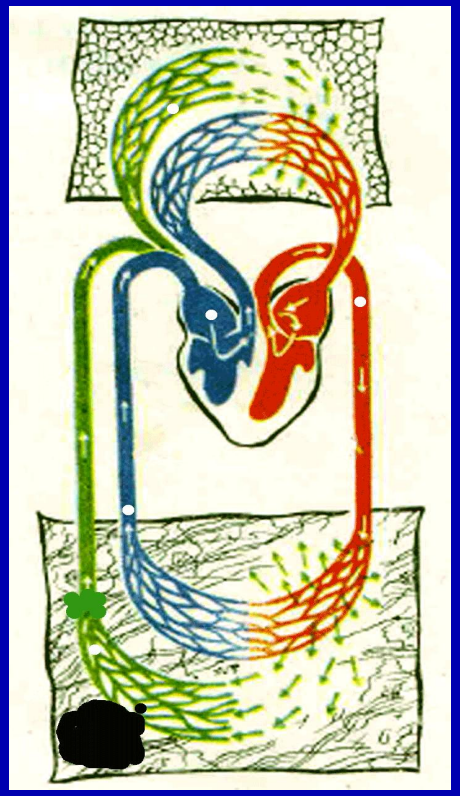
- ~ 20,000 people worldwide died of cancer every day
- Metastasis is responsible for 90% of the deaths caused by cancer

The early markers of metastasis development, cancer recurrence, and therapy efficacy:

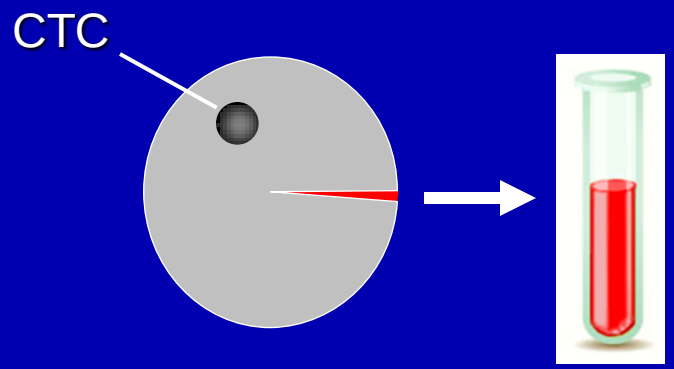
1. Blood CTCs
2. Lymph CTCs
3. Metastasis in a SLN

Circulating tumor cells (CTC) and metastasis

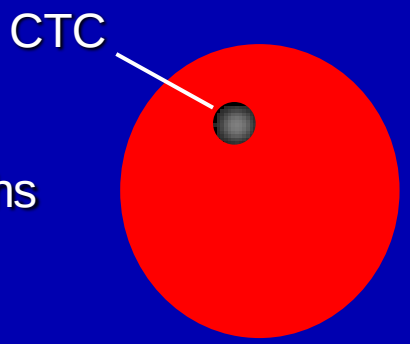




Conventional *ex vivo* blood test



***In vivo* whole blood volume test**

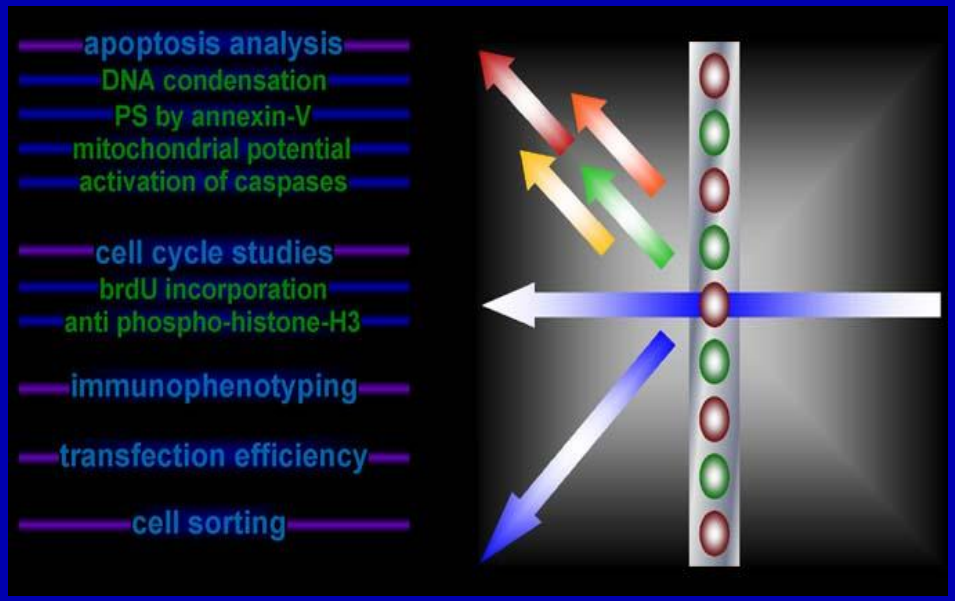


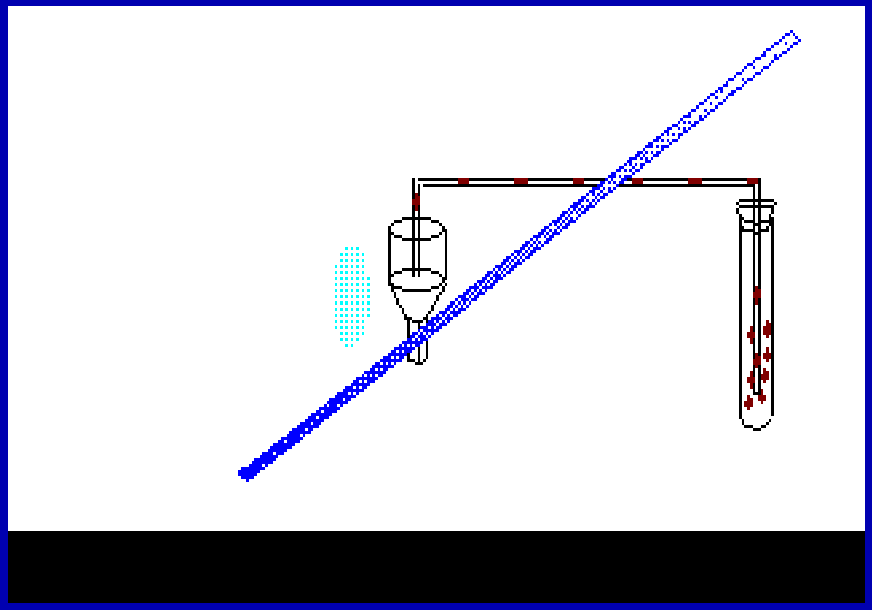
Metastasis leads to the most (up to 90%) cancer deaths

Flow Cytometry *in vitro*

Background: Flow cytometry is a well established powerful diagnostic method that revolutionized cell diagnostics *in vitro* with flow velocity of 0.1-5 m/s

Problem: The invasive extraction of cells from a living organism followed by fluorescence labeling may introduce artifacts and make it impossible to conduct long-term monitoring of the cells in the complex natural biological environment



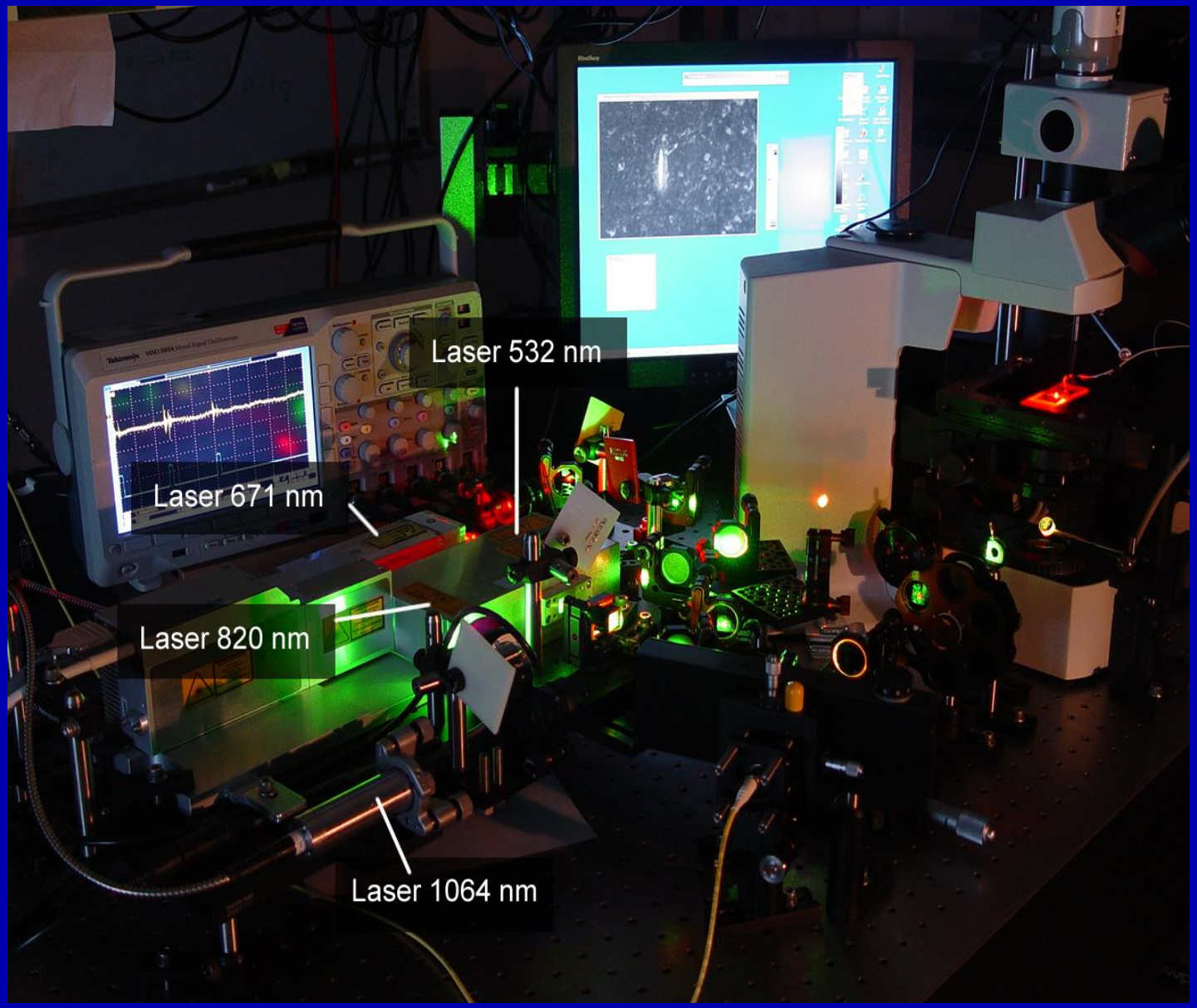


In vivo detection of CTCs

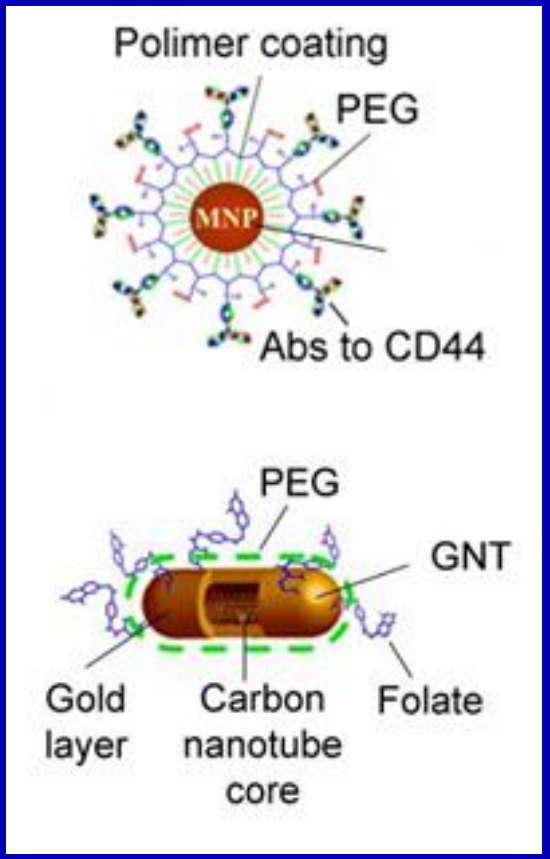
In vivo **real-time photoacoustic flow cytometry (PAFC) was developed for detection and intravascular magnetic enrichment of CTCs targeted by strongly absorbing functionalized nanoparticles as PA molecular contrast agents.**

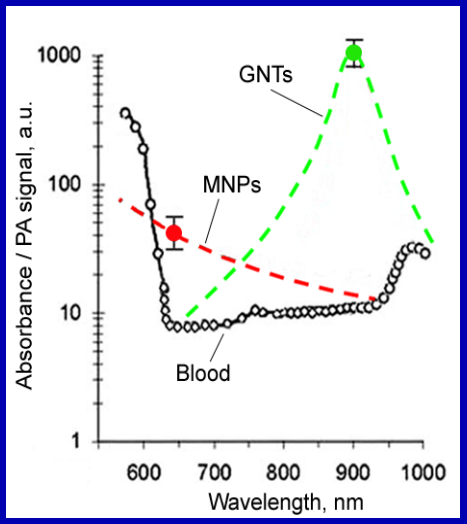
PAFC is a technology that integrates flow cytometry, nanotechnology, laser spectroscopy, and photoacoustics at single cell level.

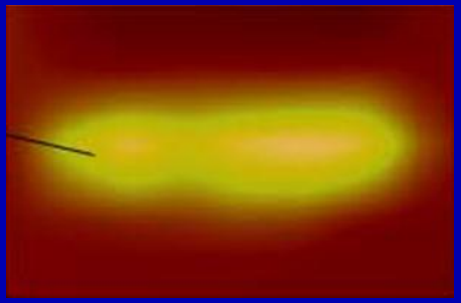
Multicolor ultra-fast photoacoustic flow cytometer



Multiplex targeting of cancer biomarkers using multicolor gold and magnetic nanoparticles





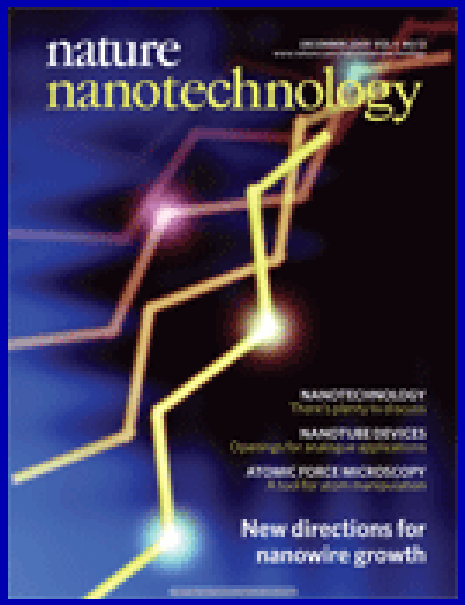


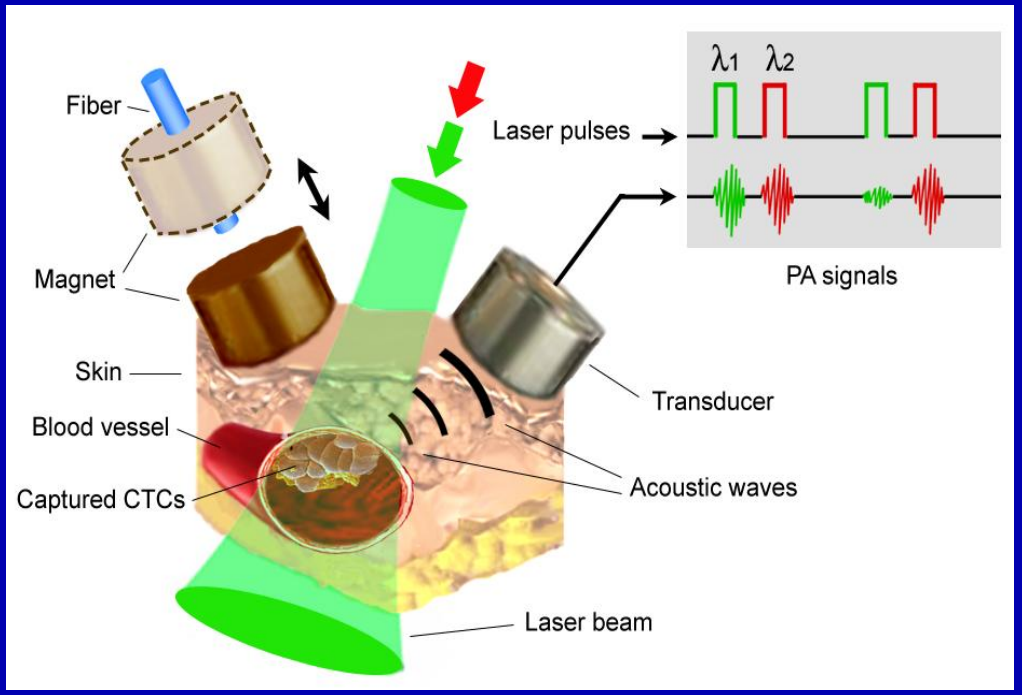
Zharov VP. Ultrasharp nonlinear photothermal and photoacoustic resonances and holes beyond the spectral limit. *Nature Photonics* 2011, published online, Jan 9.

Kim et al. Golden carbon nanotubes as multimodal photoacoustic and photothermal high-contrast molecular agents. *Nature of Nanotechnology,* 2009; 4: 688-694.

Khodakovskaya et al. PNAS 2011;108:028

Two color photoacoustic flow cytometry (PAFC) using *in vivo* magnetic enrichment of circulating tumor cells (CTCs)



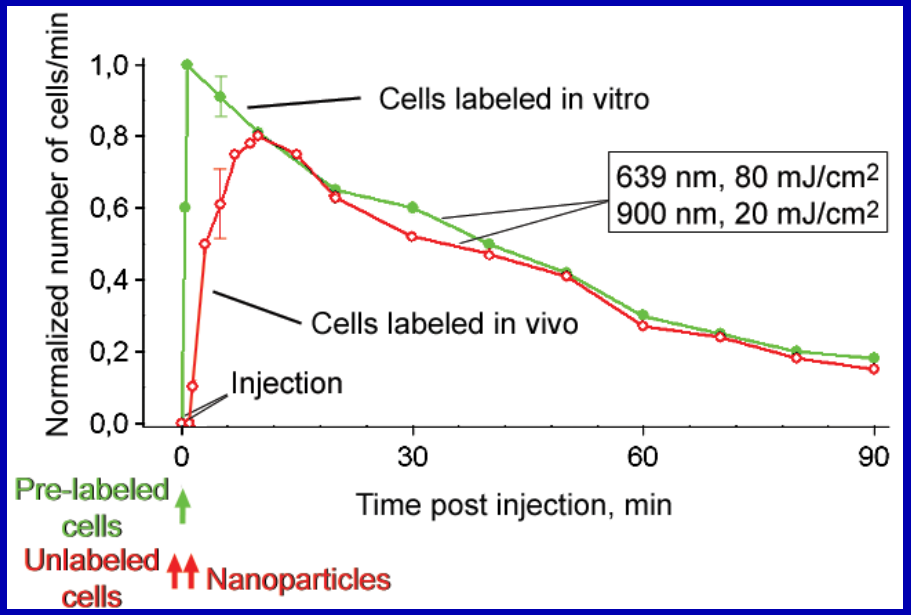


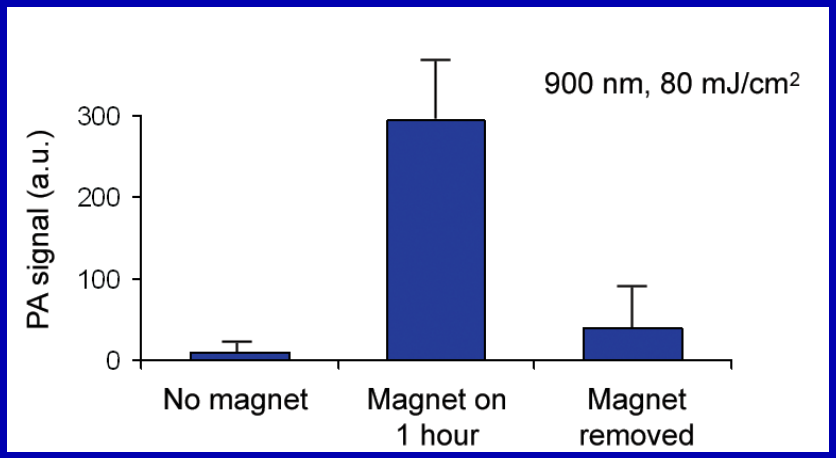
855 ***In vivo* magnetic enrichment and multiplex photoacoustic detection of circulating tumour cells**
Ekaterina I. Galanzha, Evgeny V. Shashkov, Thomas Kelly, Jin-Woo Kim, Lily Yang and Vladimir P. Zharov
→N&V p798

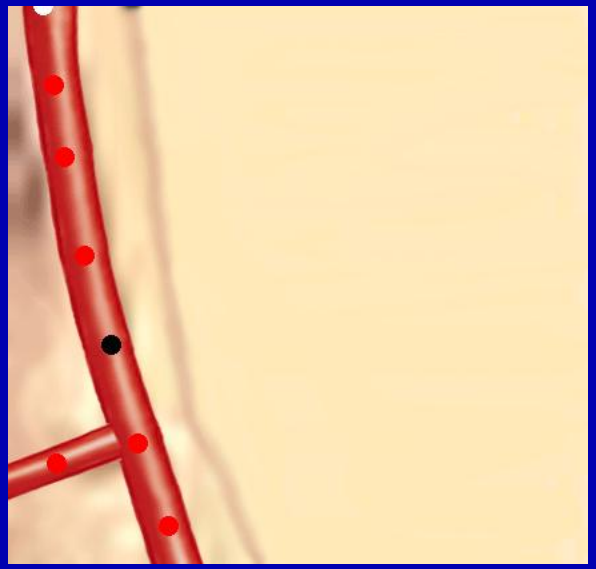
Metastatic cells are targeted by magnetic nanoparticles (MNPs) conjugated with specific antibodies

Photoacoustic blood cancer testing: diagnosis and therapy

In vivo photoacoustic flow cytometry (PAFC) and magnetic enrichment of breast cancer cells labeled by nanoparticles

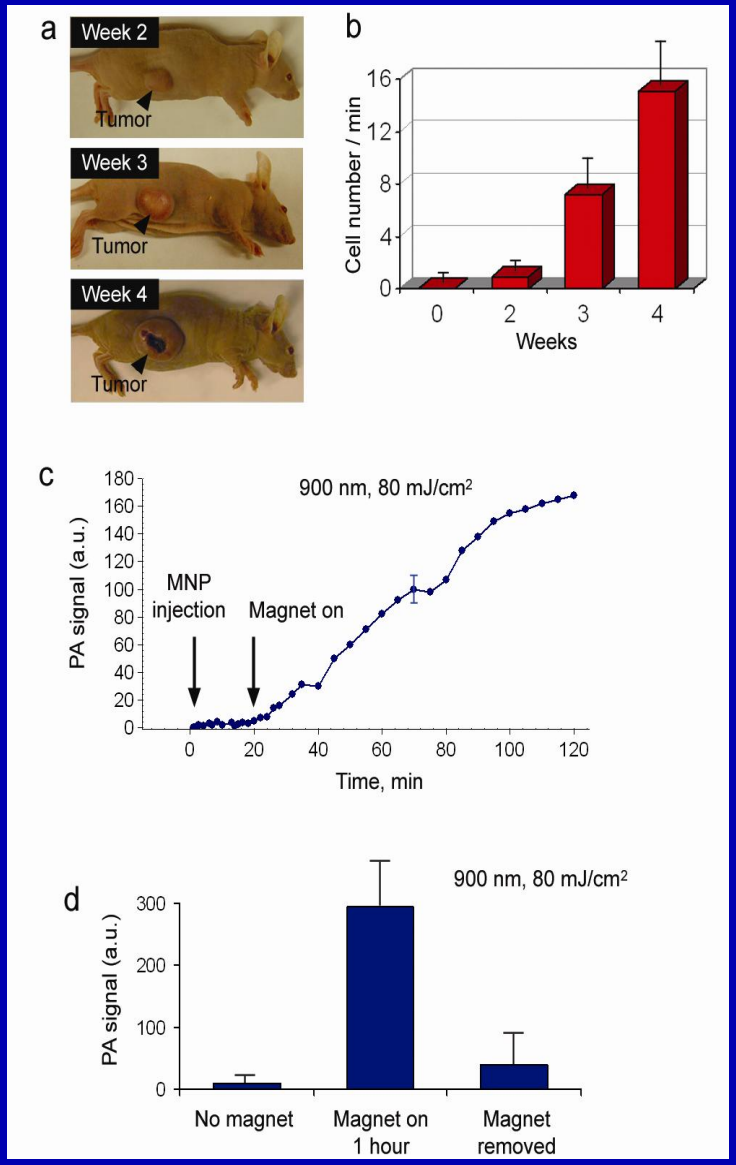




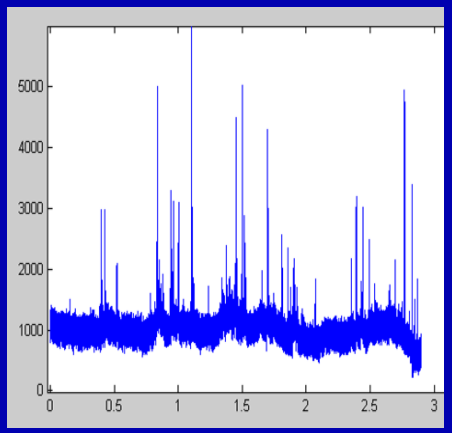


Galanzha EI, Shashkov EV, Kelly T, Kim J-W, Yang L, Zharov VP. *In vivo* magnetic enrichment and multiplex photoacoustic detection of circulating tumour cells. *Nature of Nanotechnology*, 2009, 4(12):855-60.

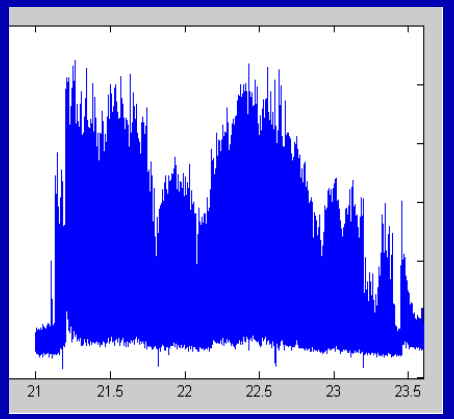
In vivo real-time magnetic capturing and photoacoustic counting of CTCs.



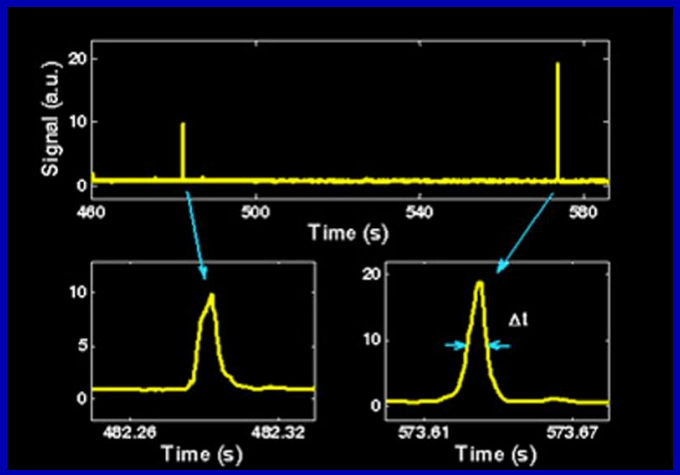
Magnet off



Magnet on



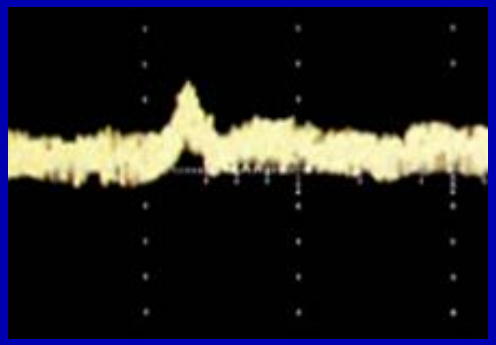
Velocity measurements



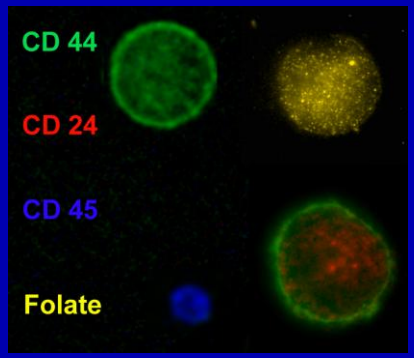
In vitro integrated photoacoustic (PA) and photothermal (PT) cytometry of cancer stem cells (CSCs)

Metastatic breast cancer cells (MDA-MB-231) were targeted by conjugated magnetic nanoparticles (MNPs) and gold carbon nanotubes (GNTs)

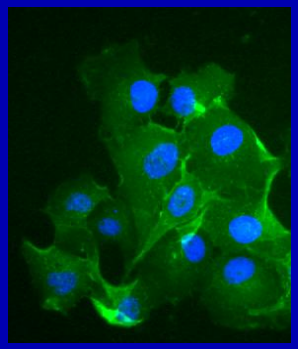
PA signal



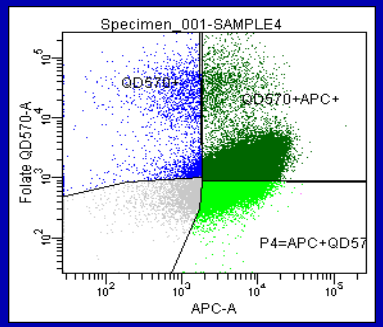
High-resolution fluorescent imaging



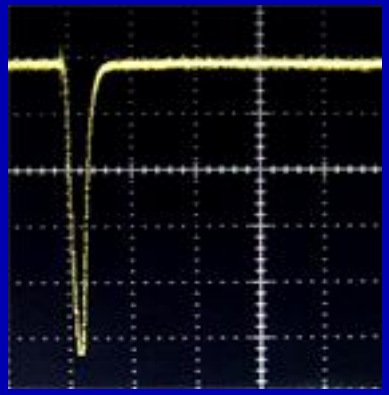
Immuno-fluorescence



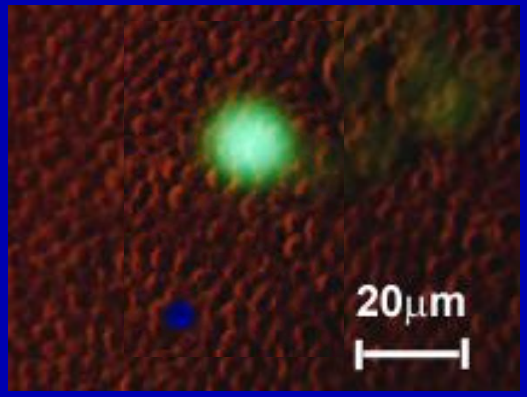
Conventional flow cytometry



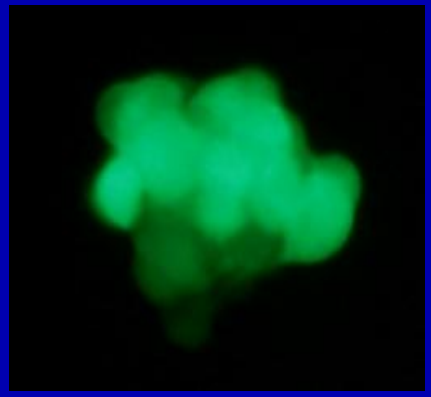
PT thermal lens signal



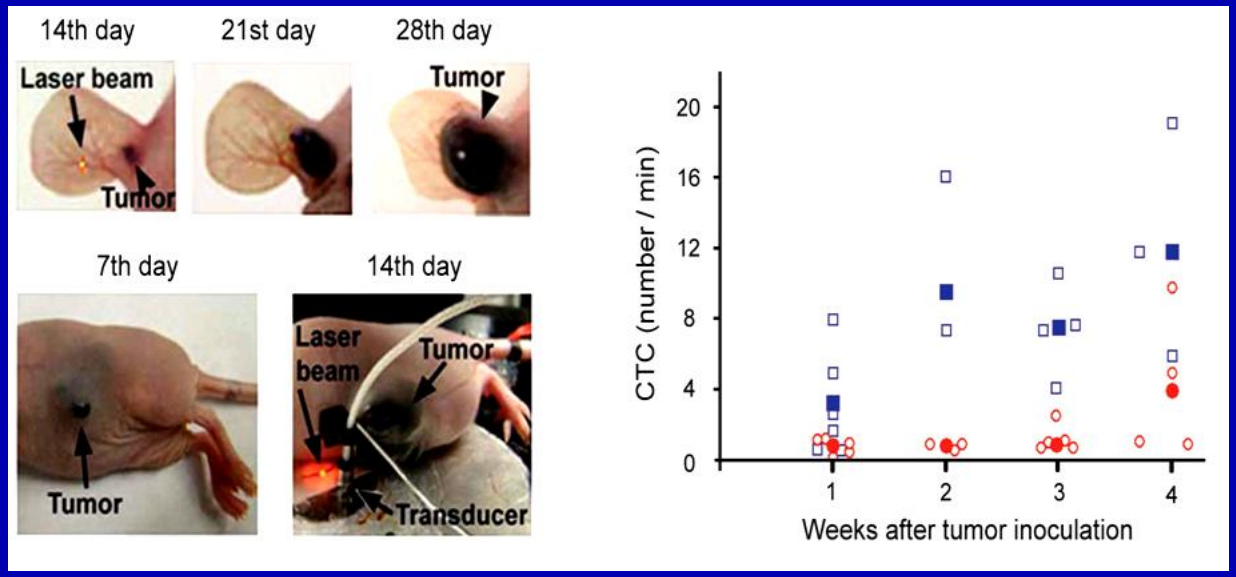
Fluorescent imaging in whole blood



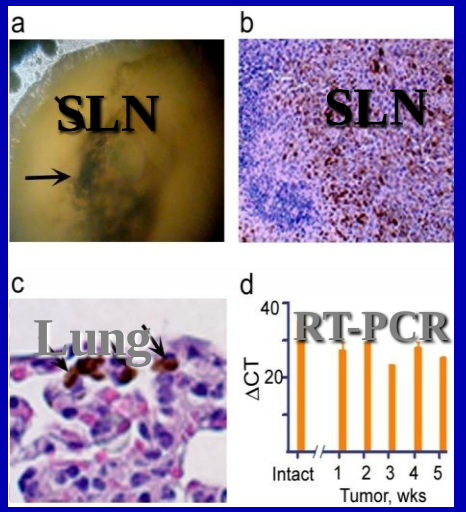
Formation of spheroids



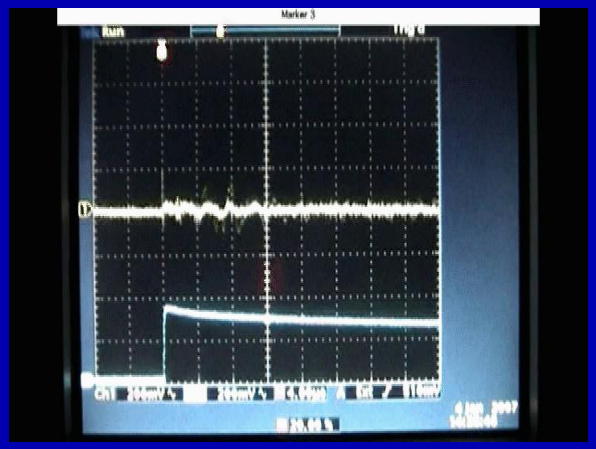
In vivo real-time detection of circulating metastatic melanoma cells (B16F10)



Micrometastasis assessment with H&E&Melan A staining and RT- PCR at 4 week



Photoacoustic signal from single melanoma cells in the circulation



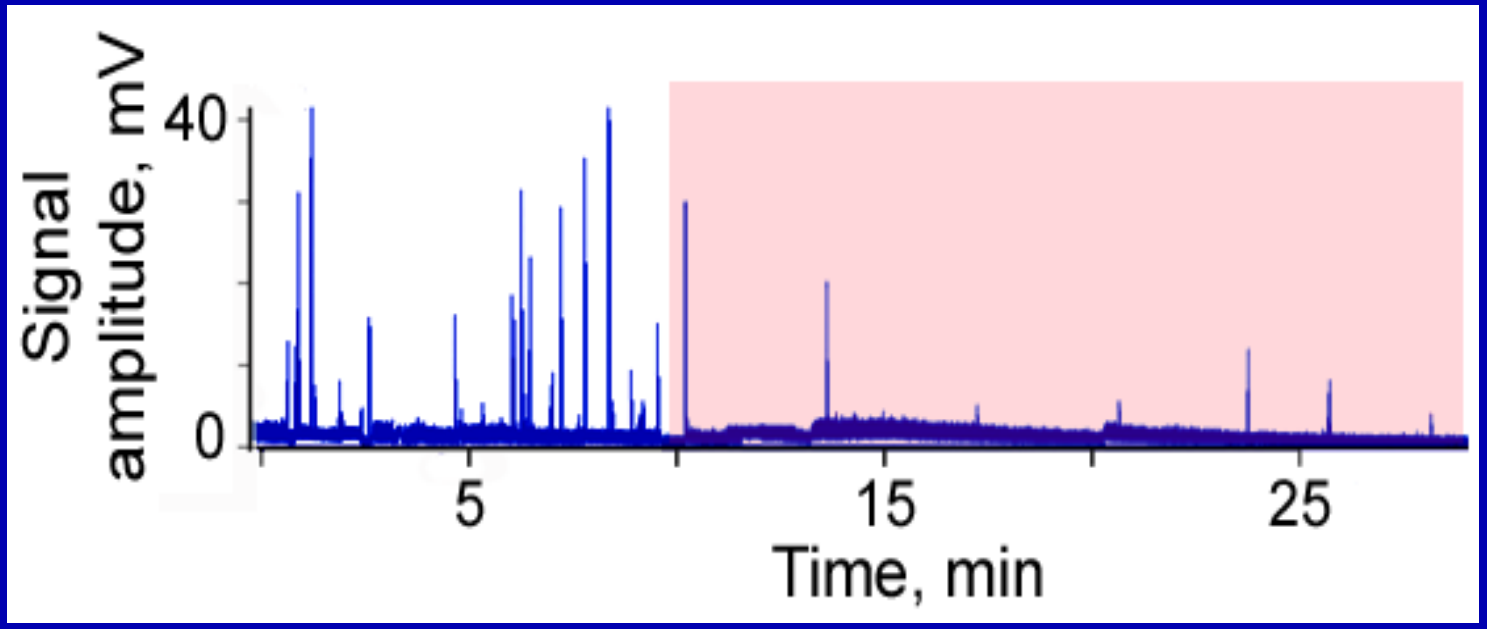
With conventional staining the evidence of few micrometastases (dimensions <70 x 40 x 40 mm) were found at week 4 only in 4 animals (n=10), in lymph nodes and one in the liver and lung.

RT-PCR analysis showed no detectable (statically significant) mRNA for Trp-1, Trp-2, and Mitf in the blood samples. The evidence of CTCs was not found with conventional hystology assays at first weeks.

Thus, at a low concentration <2-10 cells/mL, CTCs can be detected in the circulation with PA flow cytometry (PAFC) at single CTC level .

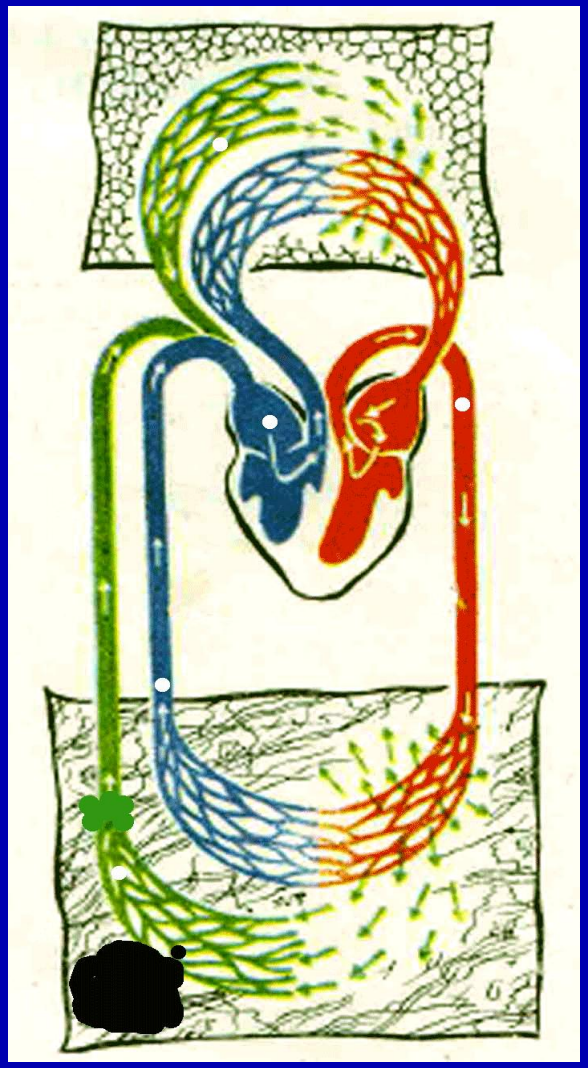
In vivo laser killing of CTCs in blood

Laser off **Laser on**



Photoacoustic lymph cancer testing: diagnosis and therapy

Diagnosis of metastasis



The early markers of metastasis development, cancer recurrence, and therapy efficacy:

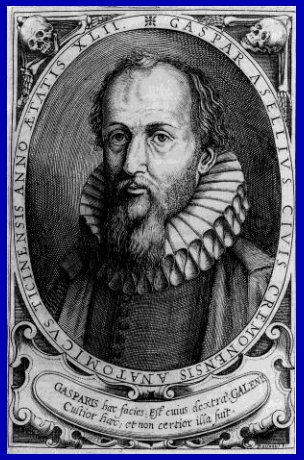
1. Blood CTCs
2. Lymph CTCs
3. Metastasis in a SLN

Limitations of existing lymph assays:

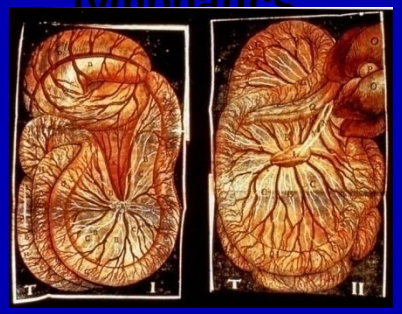
1. Lymph is more challenging substance to diagnose than blood
2. Lymph vessels are tiny colorless structures with relatively low pressure and small cell amount
3. Lymph sampling permits just a few microliters of the lymph
4. Lymph sampling requires extremely specific skills
5. Standard *in vitro* approaches to detect blood cells (e.g., in vitro flow cytometry) are impractical for diagnosis of cells in lymph flow.

XVII Century

Lymph vessels



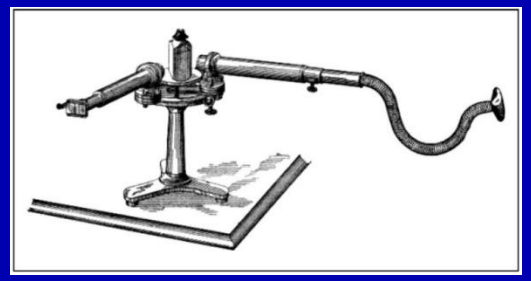
Gasparo Aselli (1581-1626): the first anatomist who described the lymphatics



XIX Century

Photothoacoustic effect

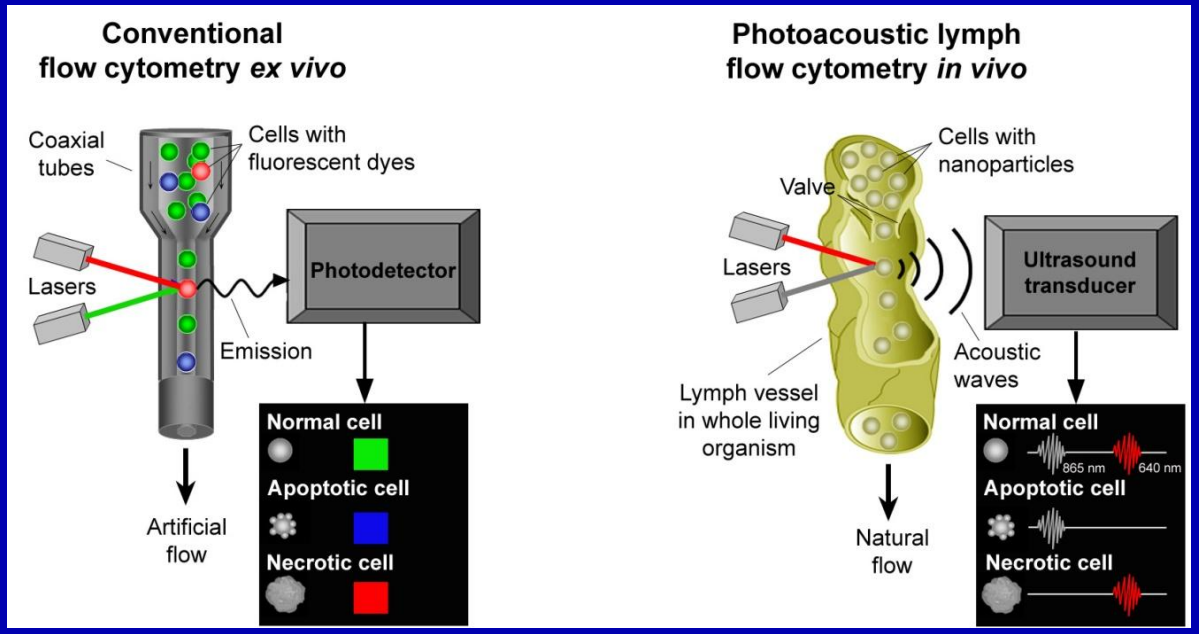
The photoacoustic effect was first reported by Alexander Bell in 1880

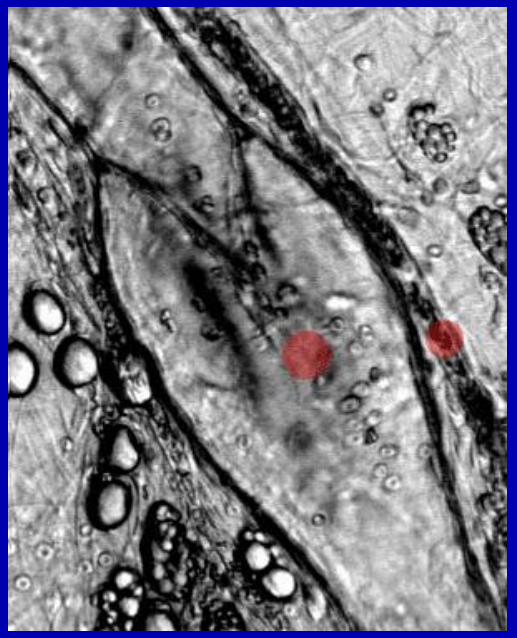


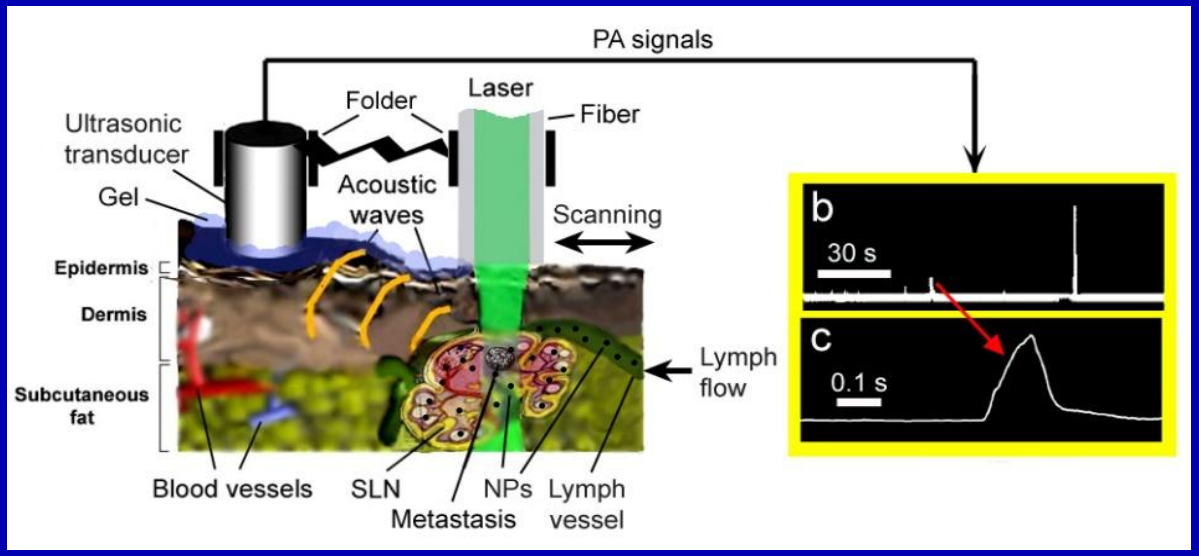
XXI Century

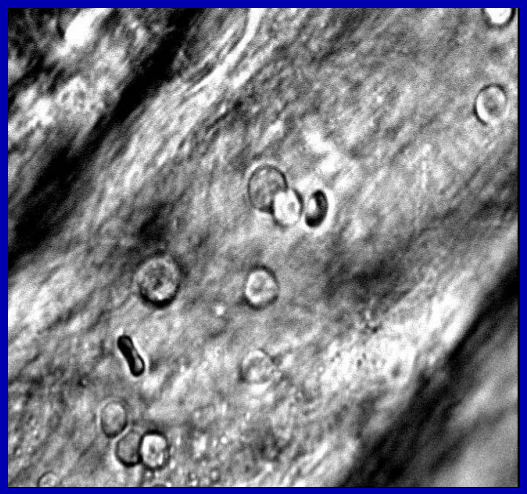
Application photoacoustic and photothermal technologies for diagnosis and therapy of lymph vessels

Multicolor photoacoustic lymph flow cytometry *in vivo*





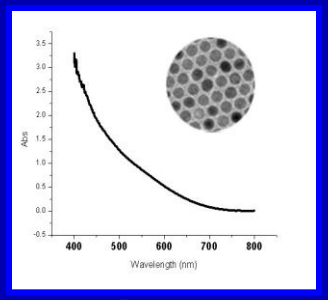




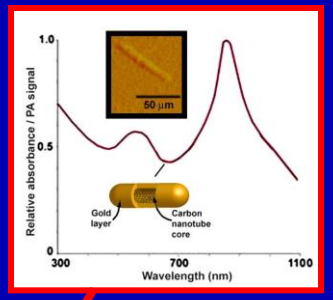
Galanzha EI, Shashkov EV, Tuchin VV, Zharov VP. In vivo multispectral photoacoustic lymph flow cytometry with natural cell focusing and multicolor nanoparticle probes. *Cytometry*, 2008; 73:884-894

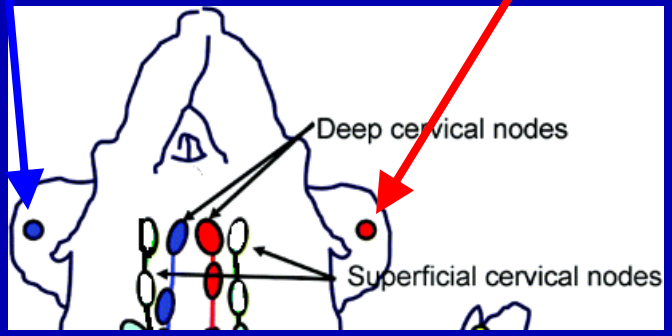
Multicolor PA Lymphography

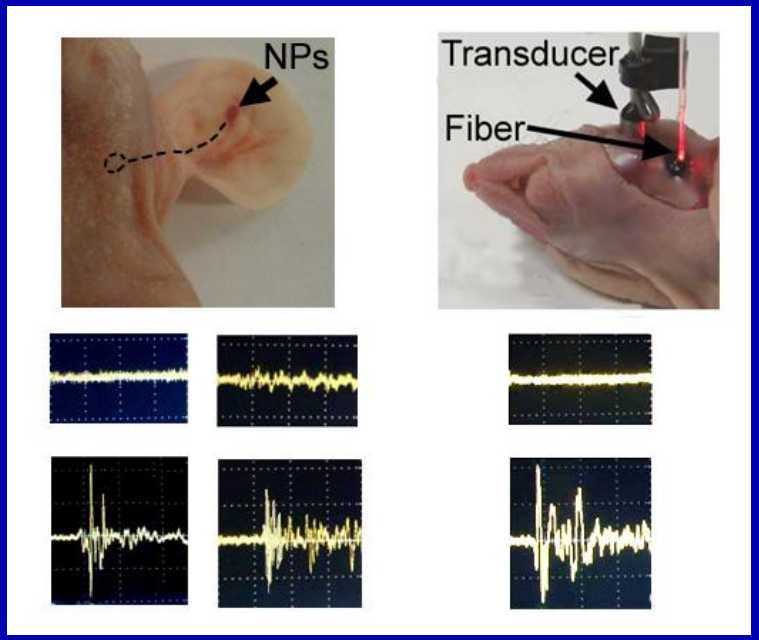
MNPs



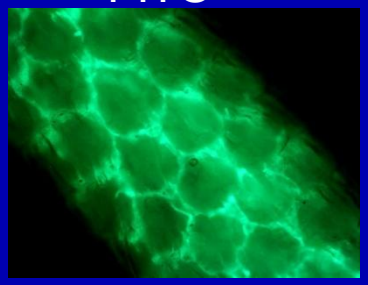
GNTs





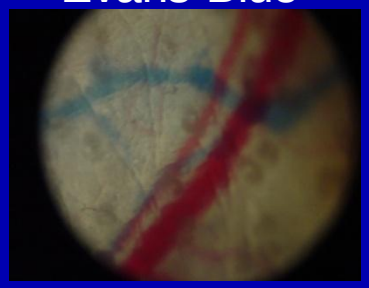


FITC



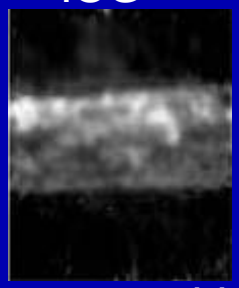
Mouse tail

Evans Blue



Mouse ear

ICG



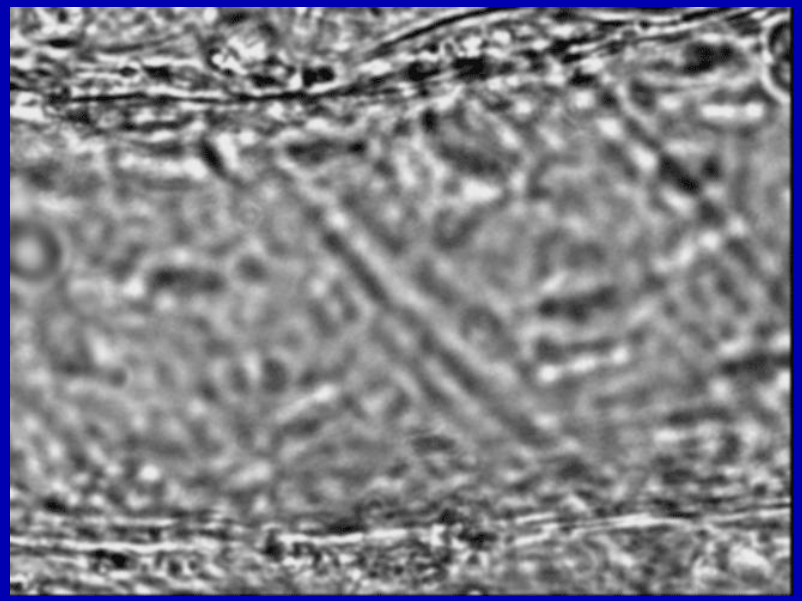
Mouse skin

MNPs and GNTs (~10^{11} NPs in 10 μl of PBS) were injected into left and right mouse ears (n=4). Both NPs quickly entered into lymphatics and migrated in less than 5 minutes to left and right cervical lymph nodes, respectively

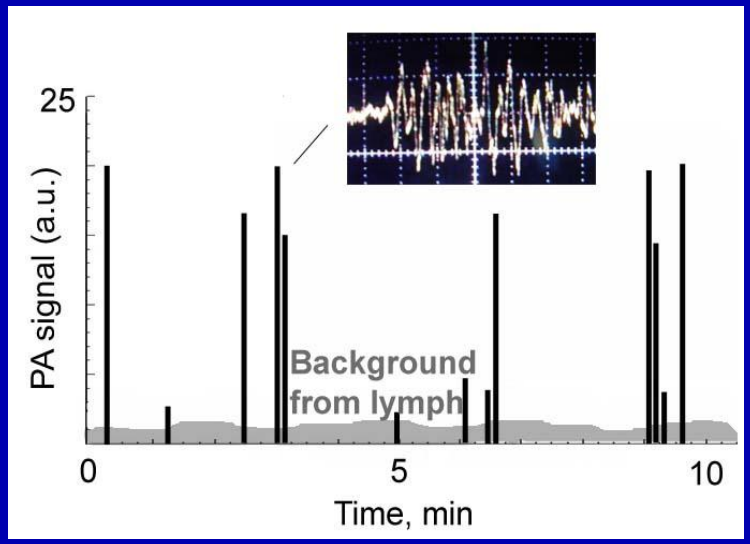
E.I. Galanzha, M.S. Kokoska, E.V. Shashkov, J-W. Kim, V.V. Tuchin, V.P. Zharov. In vivofiber-based multicolor photoacoustic detection and photothermal purging ofmetastasis in sentinel lymph nodes targeted by nanoparticles. J Biophotonics. 2, 528-539 (2009).

Photoacoustic lymph flow cytometry *in vivo*

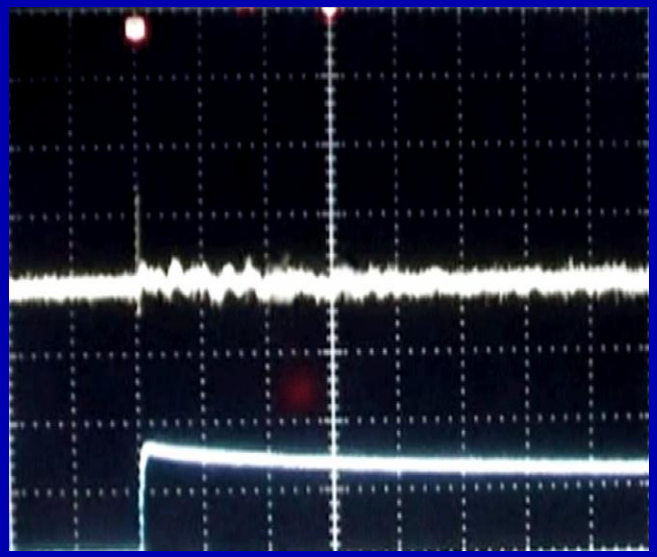
Optical imaging



2 weeks of melanoma development; primary tumor was developed in mouse ear

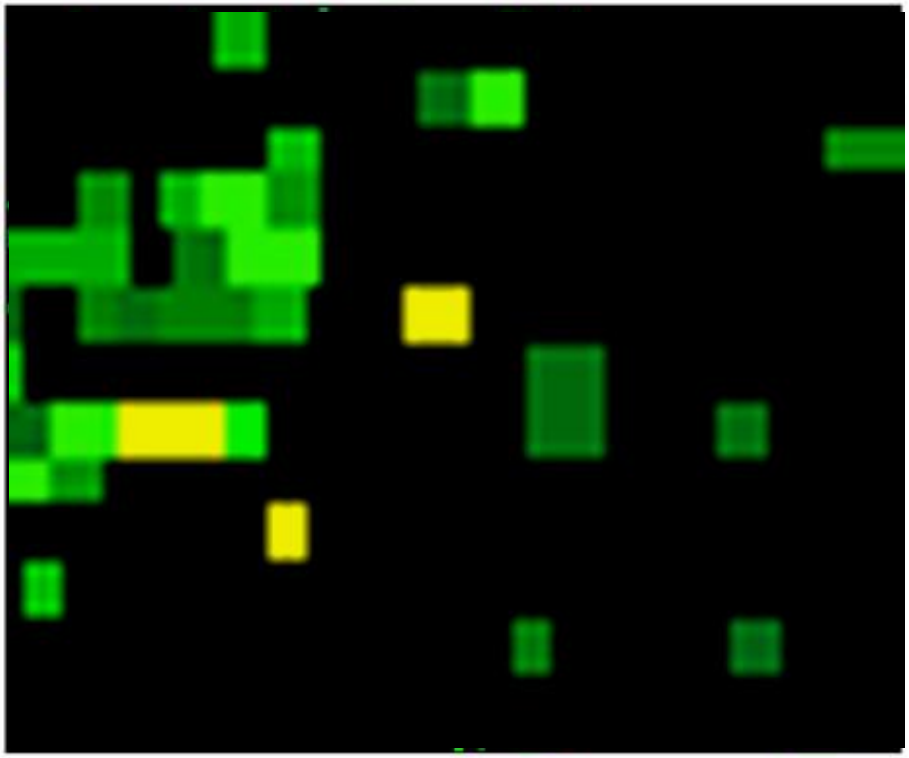


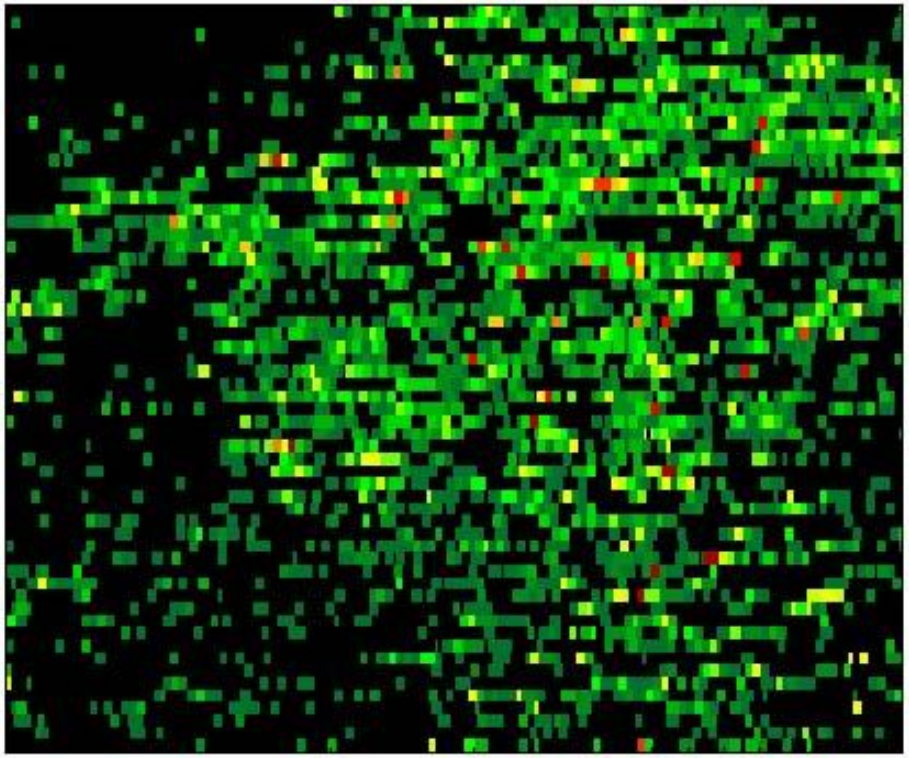
PA signals



Laser parameters: 840 nm, fluence 100 mJ/cm^2

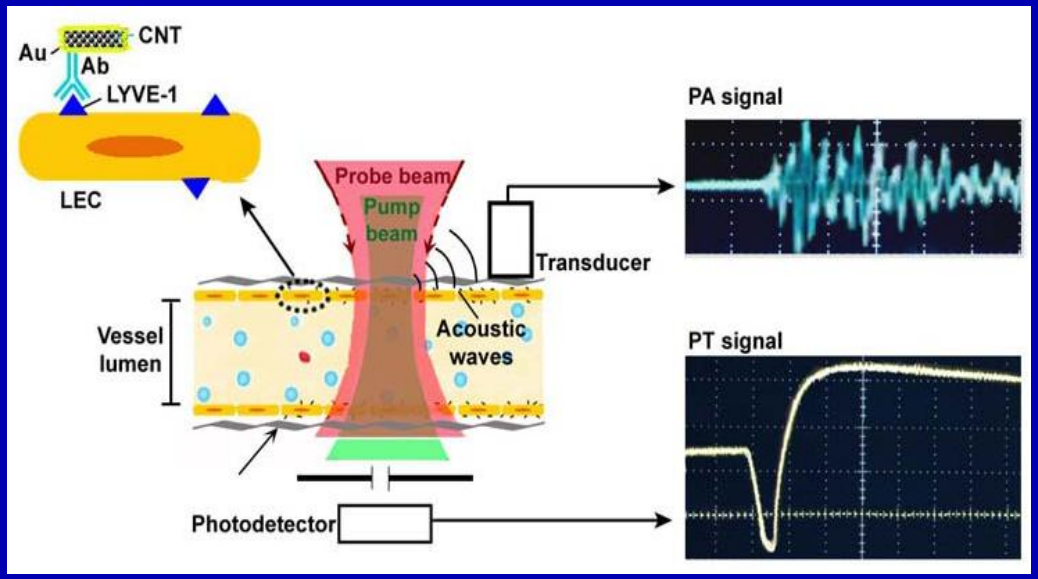
The threshold sensitivity is a one tumor cell in one million of normal lymph cells !



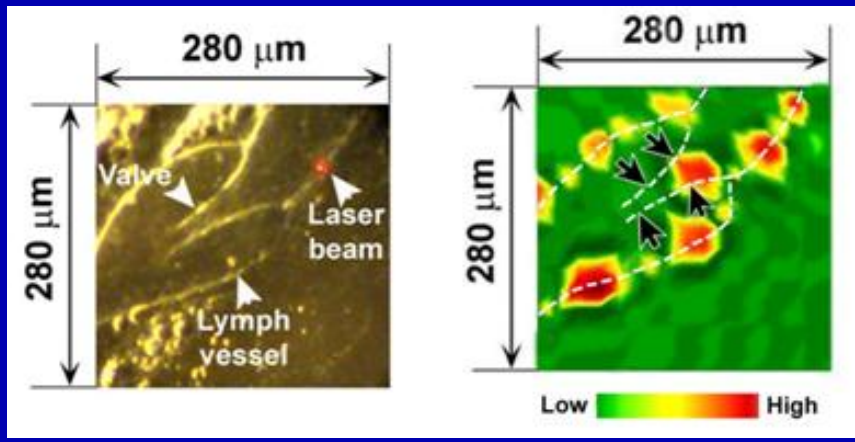


Molecular PA mapping of lymphatic endothelial cells and their targeted PT therapy

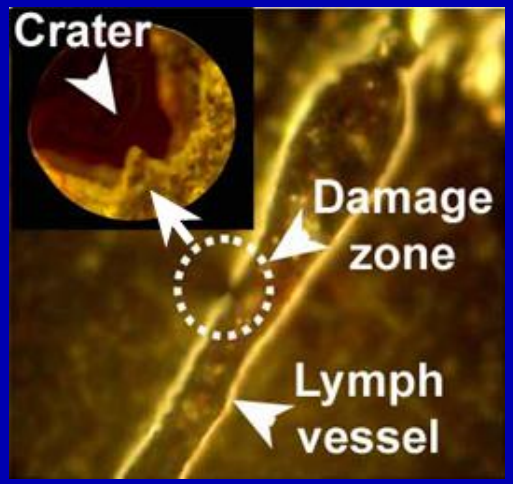
PA and PT contrast agents - golden carbon nanotubes (GNTs) bioconjugated with antibodies against LYVE-1 receptors that are expressed by lymphatic endothelial cells



Molecular detection

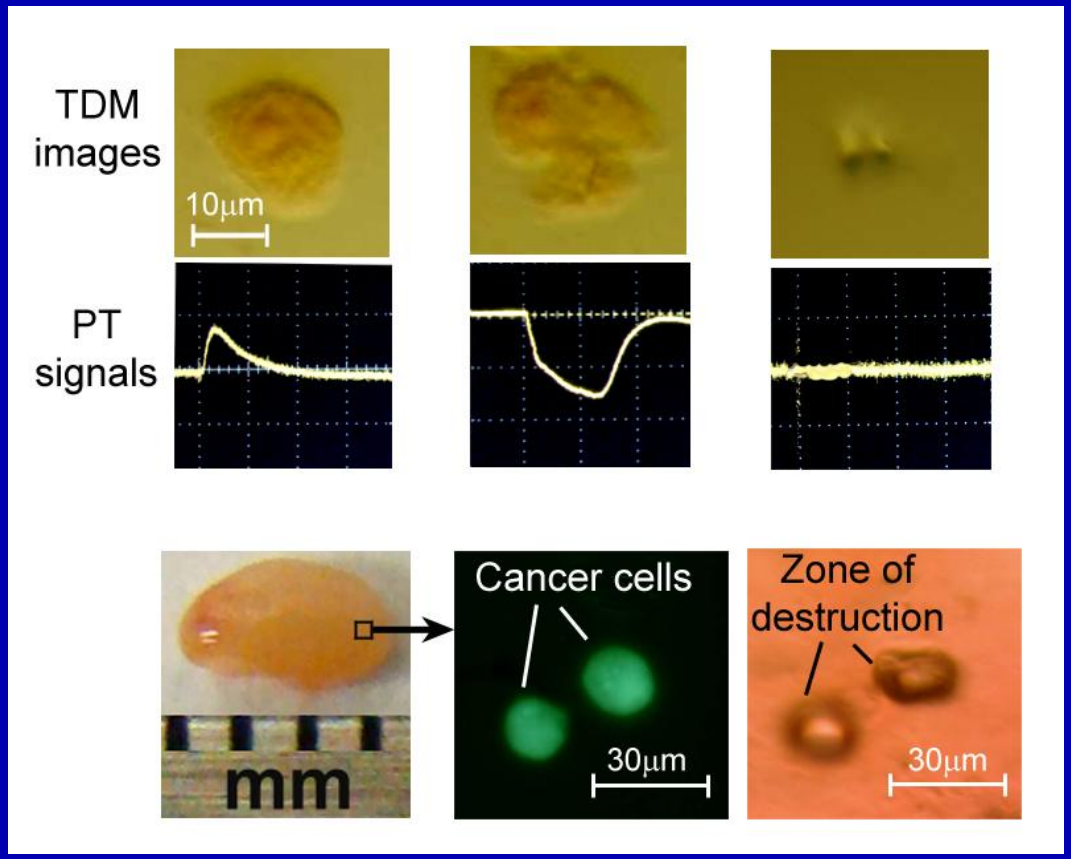


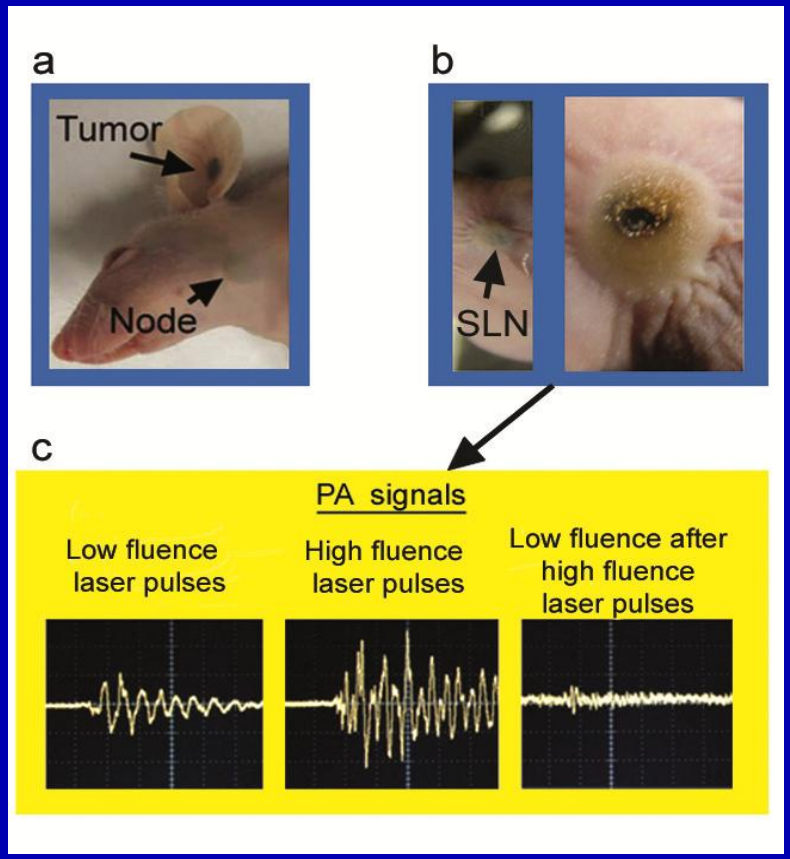
Targeted ablation



J.-W. Kim, E.I. Galanzha, E.V. Shashkov, H.-M. Moon, V.P. Zharov, Golden carbon nanotubes as multimodal photoacoustic and photothermal high-contrast molecular agents. Nature of Nanotechnology 2009;4:688-694.

In vivo targeted therapy of metastasis and estimation of therapeutic efficacy





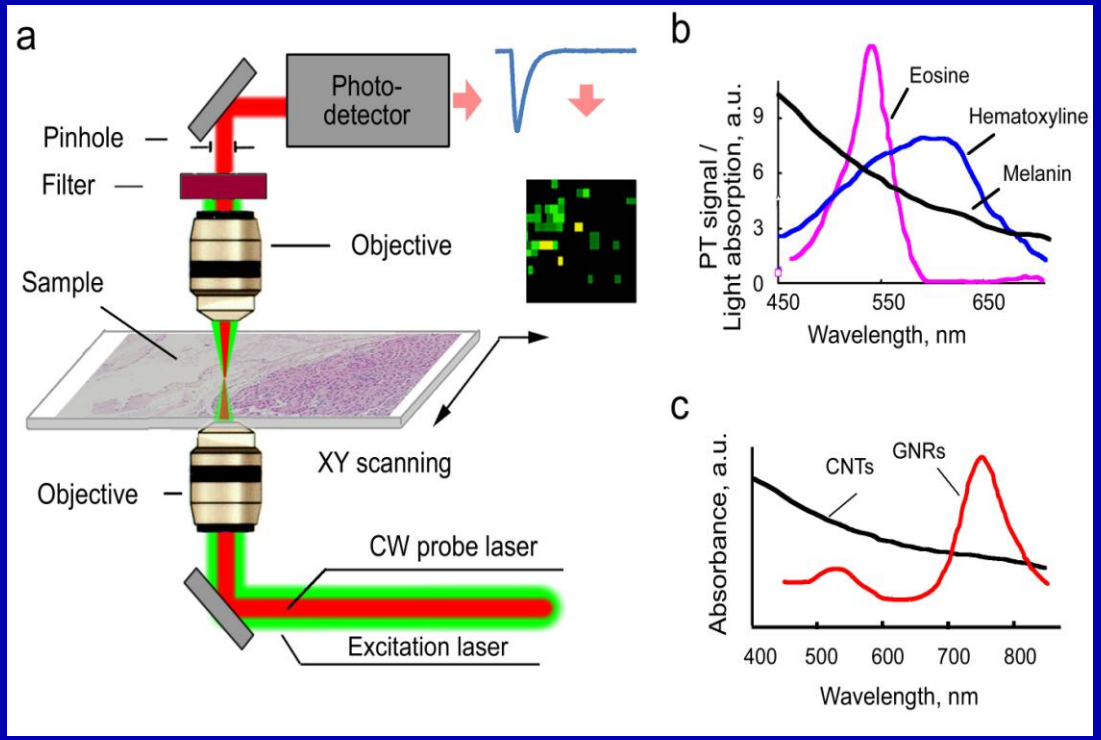
E.I. Galanzha, M.S. Kokoska, E.V. Shashkov, J-W. Kim, V.V. Tuchin, V.P. Zharov. In vivo fiber-based multicolor photoacoustic detection and photothermal purging ofmetastasis in sentinel lymph nodes targeted by nanoparticles. J Biophotonics. 2, 528 (2009).

J.-W. Kim, E.I. Galanzha, E.V. Shashkov, H.-M. Moon, V.P. Zharov, Golden carbon nanotubes as multimodal photoacoustic and photothermal high-contrast molecular agents. *Nature of Nanotechnology* 2009;4:688-694.

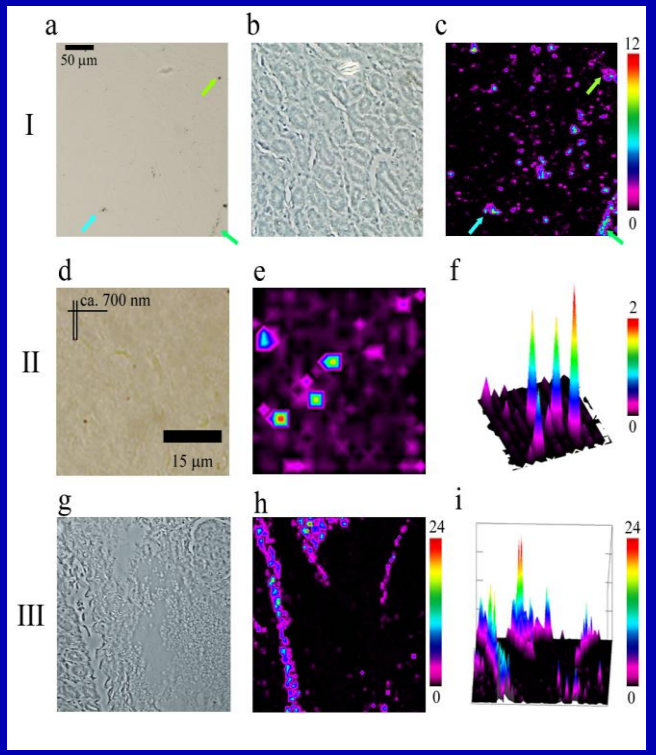
Other applications

Photoacoustic spectral scanning cytometry of histological samples

Integrated photoacoustic, photothermal, fluorescent, and transmission image cytometry

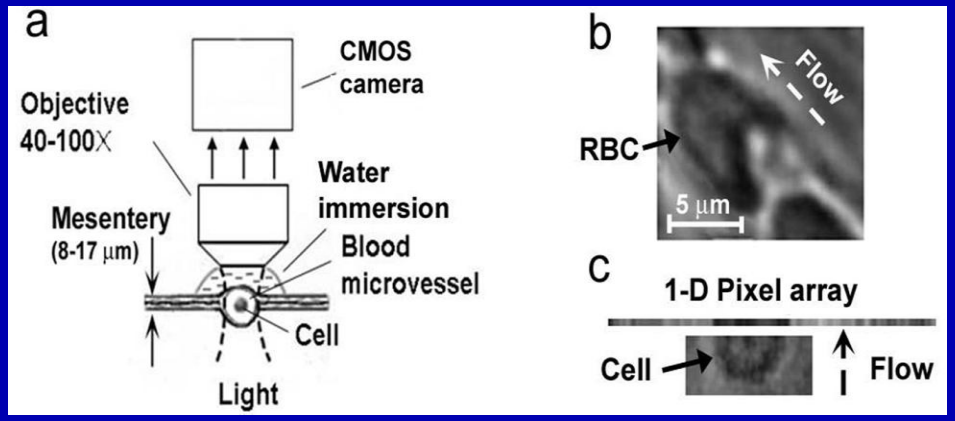


Photoacoustic mapping of carbon nanotubes in lung of tumor –bearing mouse

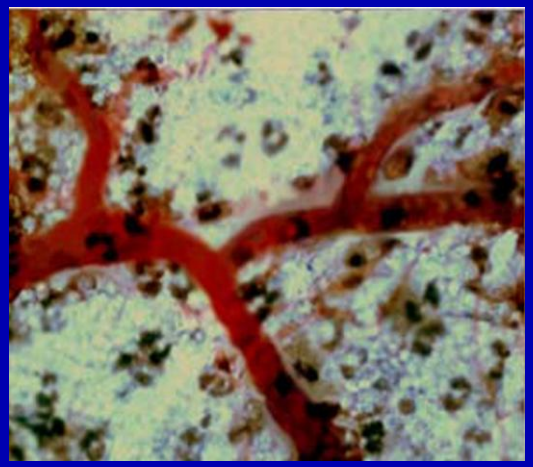


Cytometry A, 2010

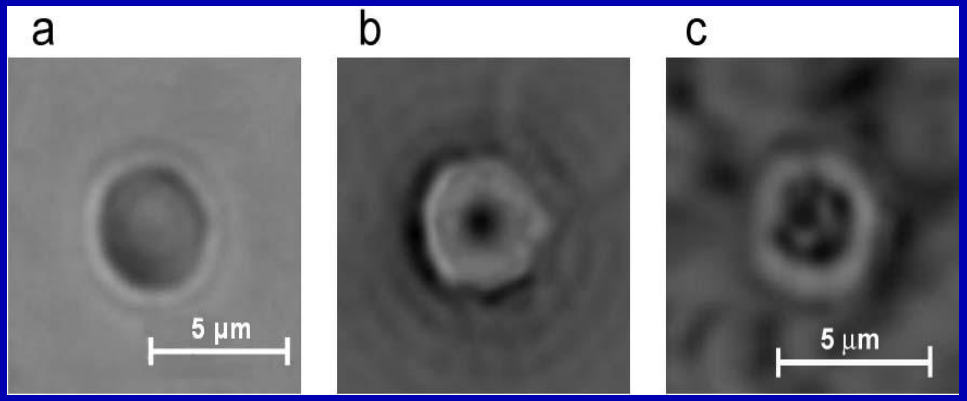
In vivo high speed (40000 frames per second) multiplex thermolens/transmission high resolution (300 nm) imaging of fast moving single cells (up to 10 m/s)

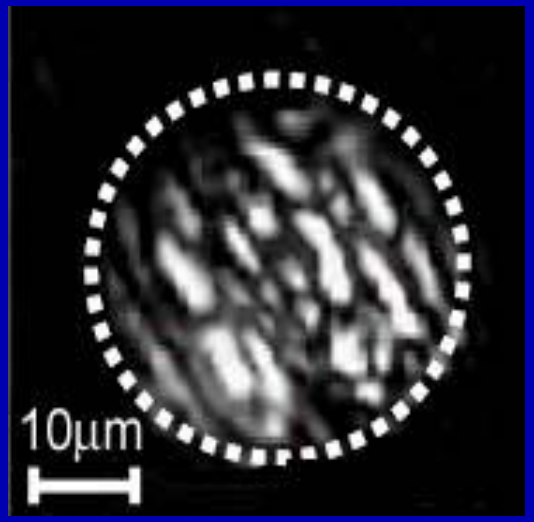


Transmission image



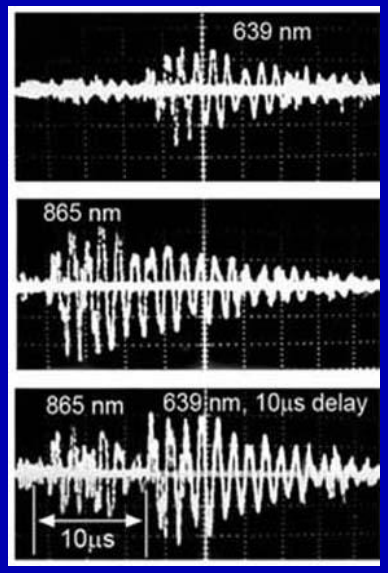
Photothermal mulitplex images of RBC at low (5 mm/s) and high (2 m/s) velocity





Far-field photothermal microscopy beyond the diffraction limit, *Opt Let*, 2003, 28:131; *In vivo* high-speed imaging of individual cells in fast blood flow. *J Biomed Opt* 2006; 11:054034; *Opt Lett*, 2005;30:628; *J Cell Biochem*, 2006 97:916 (review); *World J Gastroenterol* 2007;13:192 (review); *Cytometry* 2007; 71A:191 (review)

Multicolor high-speed PA lymph flow cytometry for detection of cells, microparticles and nanoparticles



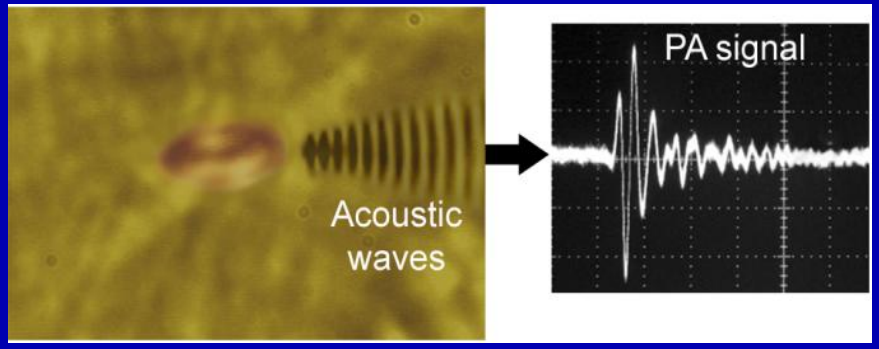
Functional status of immune-related cells

(1) Necrotic lymphocytes labeled with 16×40-nm gold nanorods (GNRs) with a maximum absorption near 640 nm,

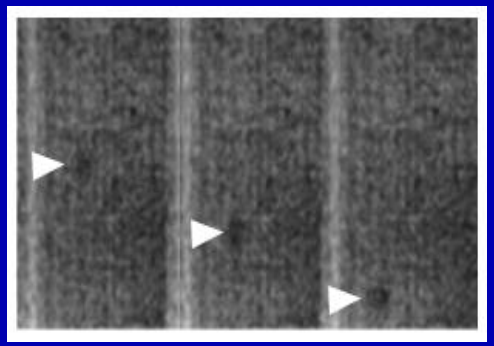
(2) Apoptotic lymphocytes labeled with gold nanoshells (GNSs) with a maximum absorption near 860 nm,

(3) Alive neutrophils labeled with 1.7×186-nm carbon nanotubes (CNTs) having monotonically decreased absorption with increases in the wavelength.

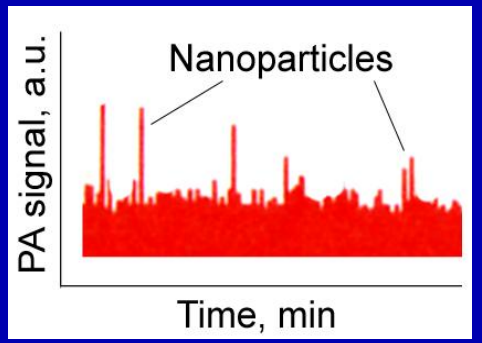
RBC



Microparticle



Nanoparticles



Conclusions

Our preclinical studies provide a new theranostic strategy to improve early diagnosis and therapy of metastatic tumors

Multicolor multiparameter photoacoustic (PA) and photothermal (PT) flow cytometry can provide *in vivo* real-time detection, enumeration, and eradication of circulating cancer stem cells directly in blood flow

1. Galanzha EI, Shashkov EV, Kelly T, Kim J-W, Yang L, Zharov VP. *In vivo* magnetic enrichment and multiplex photoacoustic detection of circulating tumour cells. *Nature of Nanotechnology*, 2009, 4(12):855-60.
2. Kim J-W, Galanzha EI, Shashkov EV, Moon H-M, Zharov VP. Golden carbon nanotubes as multimodal photoacoustic and photothermal high-contrast molecular agents. *Nature of Nanotechnology*, 2009;4:688-694.
3. Galanzha EI, Kim J-W, Zharov VP. Nanotechnology-based molecular photoacoustic and photothermal flow cytometry platform for in-vivo detection and killing of circulating cancer stem cells. *J Biophotonics*. 2009;2:725-735.
4. Galanzha EI, Shashkov EV, Spring P, Suen JY, Zharov VP. In vivo label-free detection of circulating metastatic melanoma cells by two-color photoacoustic flow cytometry. *Cancer Res*, 2009;69:7926-7934.

The advantages of *in vivo* photoacoustic and photothermal flow cytometry :

Use of the same technical platform that provides a robust integration of molecular diagnosis and targeted therapy *in vivo*

Testing of the almost entire blood volume (~5 liter) compared to conventional blood samples (5-10 mL) that provides detection and killing of rare tumor-initiating cells

Unprecedented threshold sensitivity as a one tumor cell in one billion of normal blood cells

Detection of rare cancer cell subpopulations among blood cells at the highest rate of ~10^9 cells/sec compared to ~10^5 cells/sec in conventional flow cytometry

Nedosekin et al. Ultra-fast photoacoustic flow cytometry with high pulse repetition rate 0.5 MHz nanosecond lasers. Opt Express, 2010.
Shashkov et al. Photothermal and photoacoustic flow cytometry in vitro and in vivo. Opt Express, 2010.

Conclusion

- In vivo integration of flow cytometry with nanotechnology as a new tool in the biomedical research demonstrates the unprecedented threshold sensitivity as one tumor cell in the background of one billion normal blood cells (i.e., ~ 1 CTC/mL) **and one million of normal lymph cells**
- High potential of the quick translation to humans
- Future studies include new nanoparticles (e.g., multi color), targets (e.g., different microparticles), methods (e.g., multispectral with multiplex targeting) and applications, e.g., establishing an unknown correlation of primary tumor and distant metastasis progression with the count of blood and lymphatic CTCs and sentinel lymph node (SLN) status.

1. Galanzha EI, Shashkov EV, Tuchin VV, Zharov VP. In vivo multispectral photoacoustic lymph flow cytometry with natural cell focusing and multicolor nanoparticle probes. *Cytometry*, 2008; 73:884-894
2. Zharov VP, Galanzha EI, Tuchin VV. Photothermal Image Flow Cytometry *in Vivo. Opt. Lett.* 2005;30:628-630.
2. Galanzha EI, Kokoska MS, Shashkov EV, Kim J-W, TuchinVV, Zharov VP. In vivofiber-based multicolor photoacoustic detection and photothermal purging ofmetastasis in sentinel lymph nodes targeted by nanoparticles. *J Biophotonics*. 2, 528-539 (2009).
4. Galanzha EI, Shashkov EV, Spring P, Suen JY, Zharov VP. In vivo label-free detection of circulating metastatic melanoma cells by two-color photoacoustic flow cytometry. *Cancer Res*, 2009;69:7926-7934.
5. J.-W. Kim, E.I. Galanzha, E.V. Shashkov, H.-M. Moon, V.P. Zharov, Golden carbon nanotubes as multimodal photoacoustic and photothermal high-contrast molecular agents. *Nature of Nanotechnology* 2009;4:688-694.
6. Galanzha EI, Shashkov EV, Kelly T, Kim J-W, Yang L, Zharov VP. *In vivo* magnetic enrichment and multiplex photoacoustic detection of circulating tumour cells. *Nature of Nanotechnology*, 2009; 4(12):855-60.
7. Zharov VP. Ultrasharp nonlinear photothermal and photoacoustic resonances and holes beyond the spectral limit, *Nature Photonics,* 2011;5:110-116.

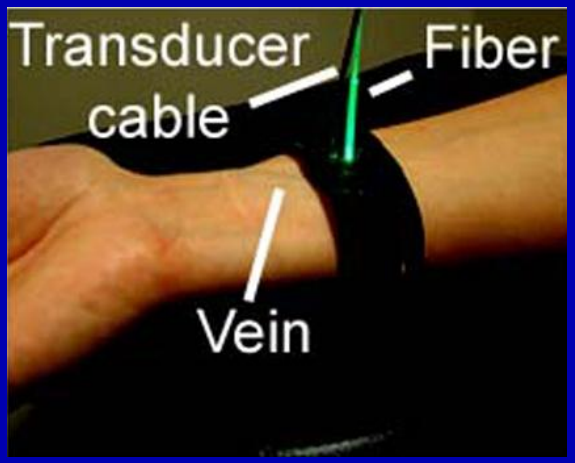
Clinical relevance

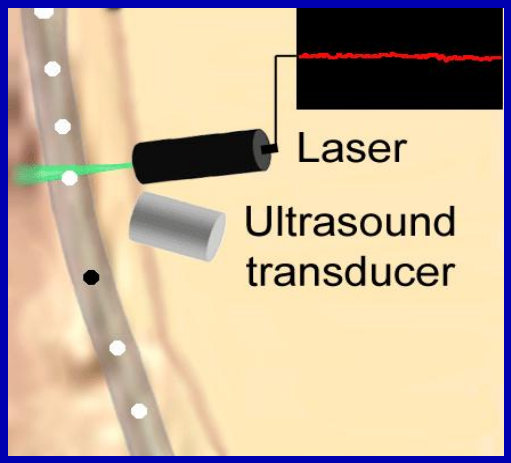
The clinical relevance of presented method is based on:

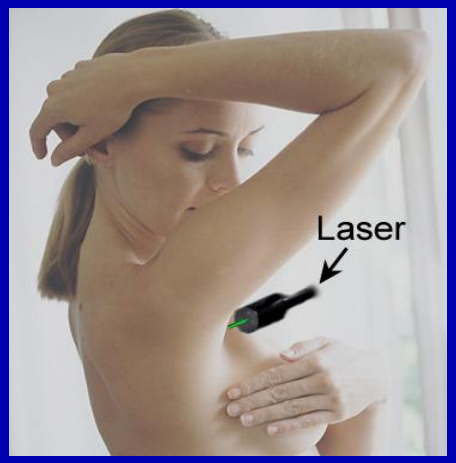
Application of the laser fluence which is well within laser safety standard for humans

Using of low toxic nanoparticles as molecular contrast agents

Easy assessment of 2-3 mm in diameter peripheral blood vessels at depth of 2-4 mm that is within well-documented capability of photoacoustics (3-5 cm).







Clinical prototypes

Acknowledgements

This work is supported by the National Institute of Health (NIH) grants:
R01 CA131164; R01 EB009230; R21 CA139373,
The National Science Foundation (NSF) grant
DBI 0852737
Department of Defense (DoD):
W88XWH-10-2-0130; BC103405; BC103391