Detection and Enumeration of Rare Circulating Cells with In Vivo Flow Cytometry

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I) *In Vivo* Flow Cytometry (IVFC):
What is it and why is it useful?

II) Our work in <u>High-Sensitivity</u> *IVFC*
"Computer Vision In Vivo Flow Cytometry"
"Diffuse Fluorescence In Vivo Flow Cytometry"

Counting Circulating Cells

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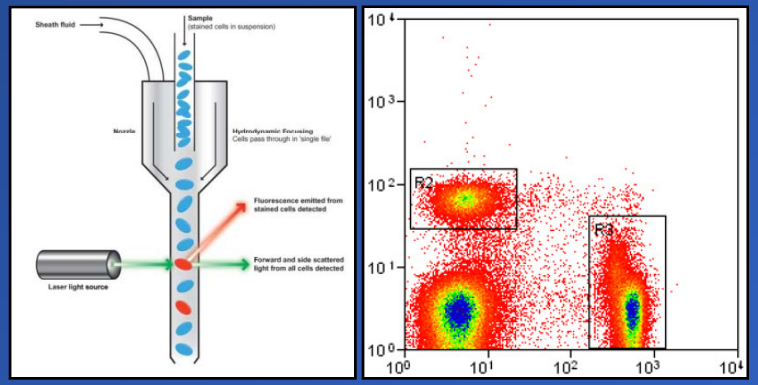
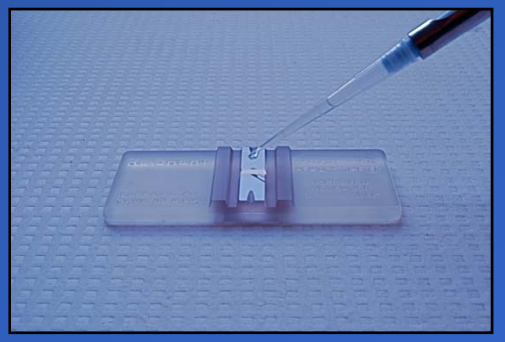
There are many applications in biomedical research where it is desirable to know the number of a specific type of cell in circulation

e.g. cancer metastasis, organ and tissue transplant biology, HIV-AIDS, immune system, hematopoietic stem cells…

Blood sample



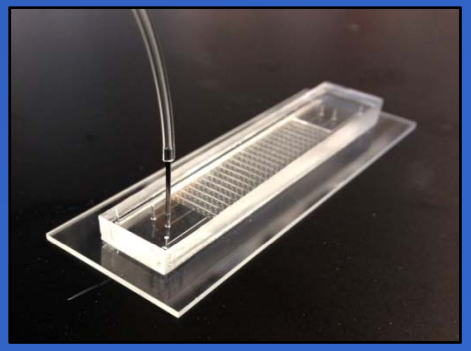
Flow cytometry



Hemocytometry

Limitations?

- Frequency of sampling (survival experiments)
- Sensitivity (blood sample volume)
- Storage, handling, enrichment of samples



Microfluidics

In Vivo Flow Cytometry

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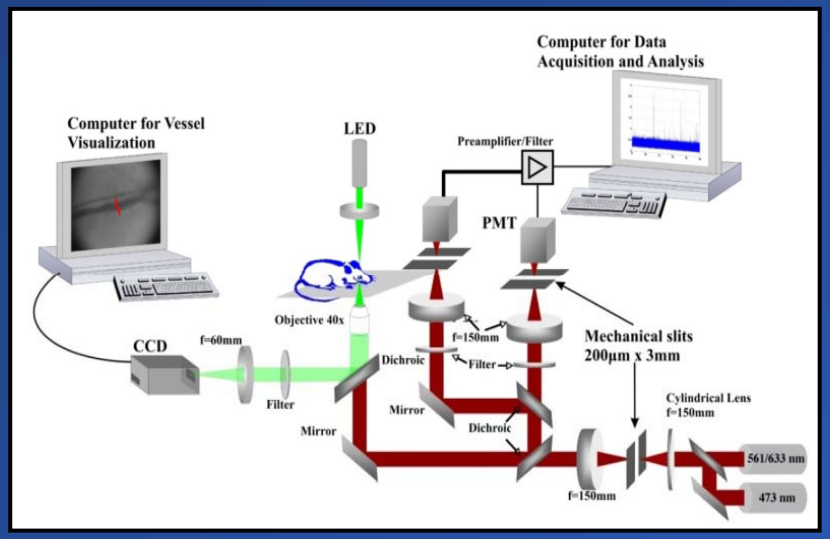
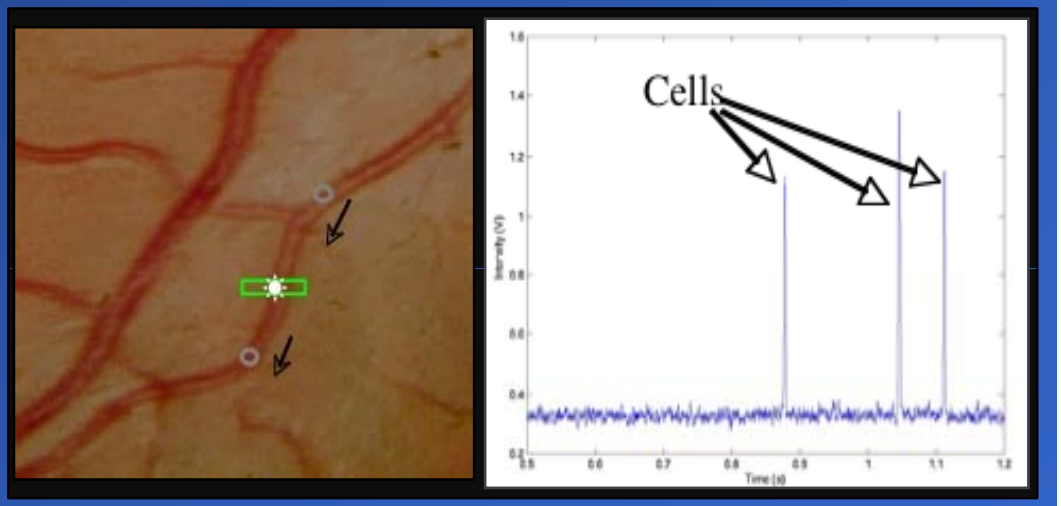
Fluorescence microscopy IVFC; mouse ear or retina
Continuous, non-invasive measurements





**Irene Georgekoudi & Charles Lin, Cancer Research 2004*

Applications of IVFC:

In vivo study of red blood cells, T-Lymphocytes, prostate cancer, breast cancer, melanoma, mesenchymal stem cells, multiple myeloma, etc.

Other IVFC Designs:

Two photon, multi-color, photo-acoustic, photothermal, etc.

CP Lin, I Georgekoudi, VP Zharov, VV Tuchin, TB Norris, X Wei, etc.

Cell Labeling in IVFC

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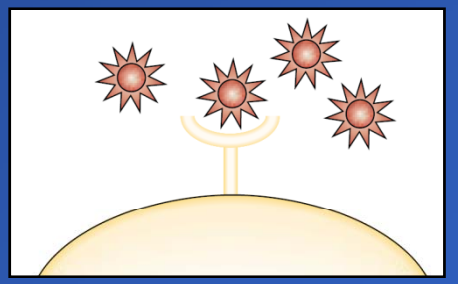
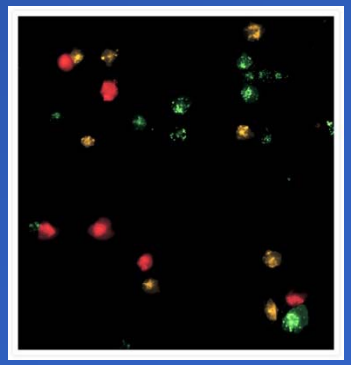
How are cells fluorescently-labeled?



1) Receptor-targeted injected probes

Fluorescent IgG antibodies or
Fab antibody fragments



2) "Ex-vivo" labeling of target cell lines

Membrane dyes, Vybrant DiD; DiR; DiL

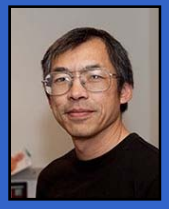
3) Fluorescent Proteins

eGFP, YFP, mCherry, etc

Pitsillides, C.M. et. al., "Cell labeling approaches for fluorescence-based in vivo flow cytometry," Cytometry A. Oct 2011; 79(10): 758–765.

IVFC Example Application: Mobilization Therapy for Multiple Myeloma

Irene Ghobrial
DFCI-HMS

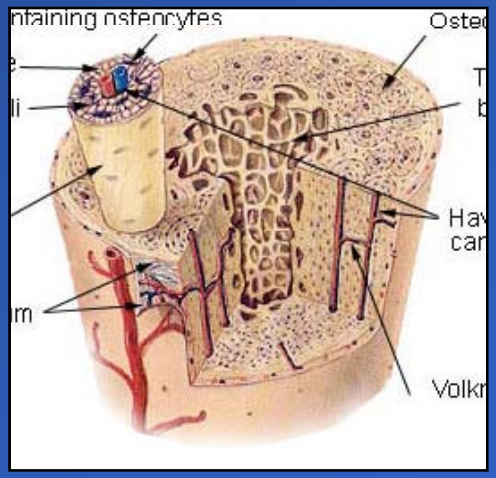


Charles Lin
MGH-HMS

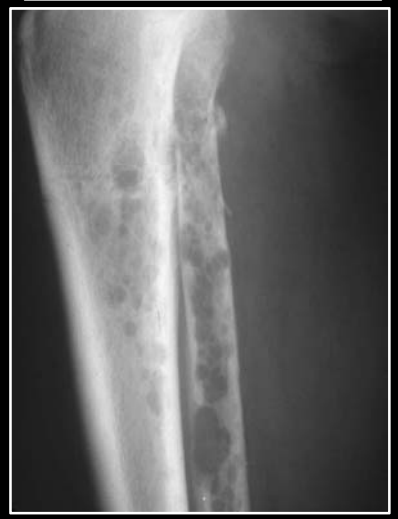
Multiple Myeloma (MM)

- Incurable blood malignancy
- Presents as multiple bone lesions
- Develop at single site, disseminates continuously via the blood stream to bone marrow niche

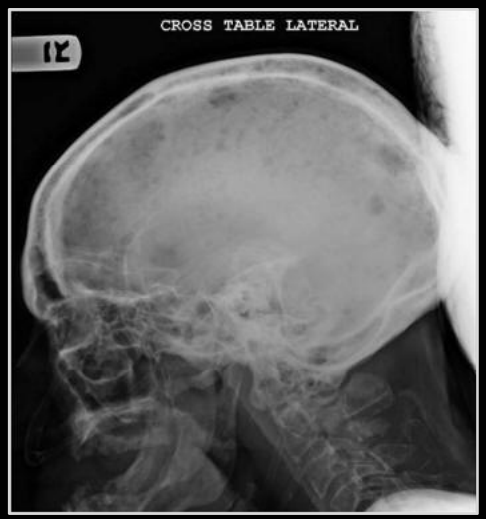
Chemotherapy (Bortezomib) is less effective on MM in bone marrow niche versus circulating MM cells



MM Tibia/fibula X-ray



MM Skull X-ray



Tx Strategy: Keep MM Cells In Circulation

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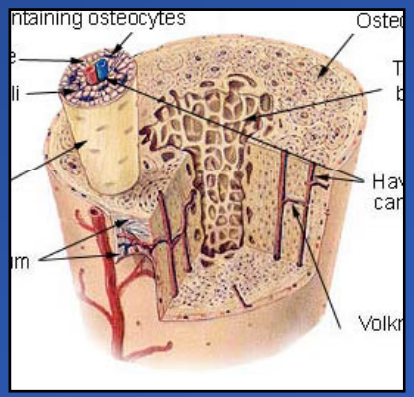
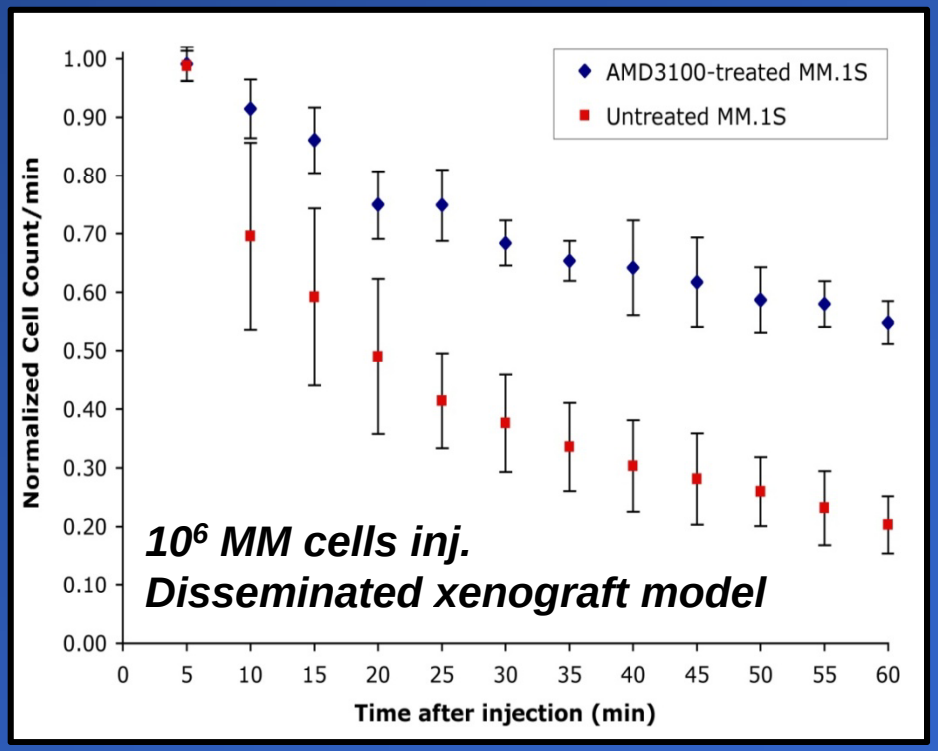
The idea: interfere with homing and adhesion of MM cells in bone marrow niches

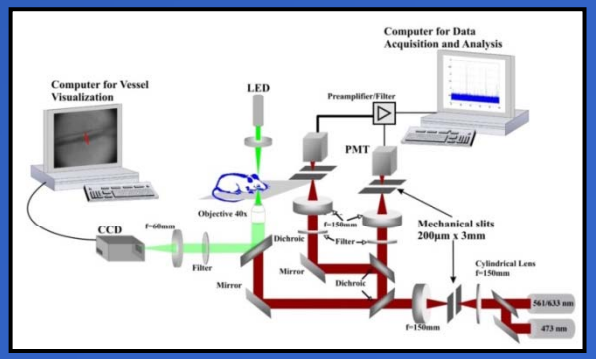
"mobilization therapy"

AMD3100; CXCR-4 inhibitor





10^6 MM cells inj.
Disseminated xenograft model



AK Azab et. al.

CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy

BLOOD, 30 APRIL 2009 • VOLUME 113, NUMBER 18

"Bench to Bedside"

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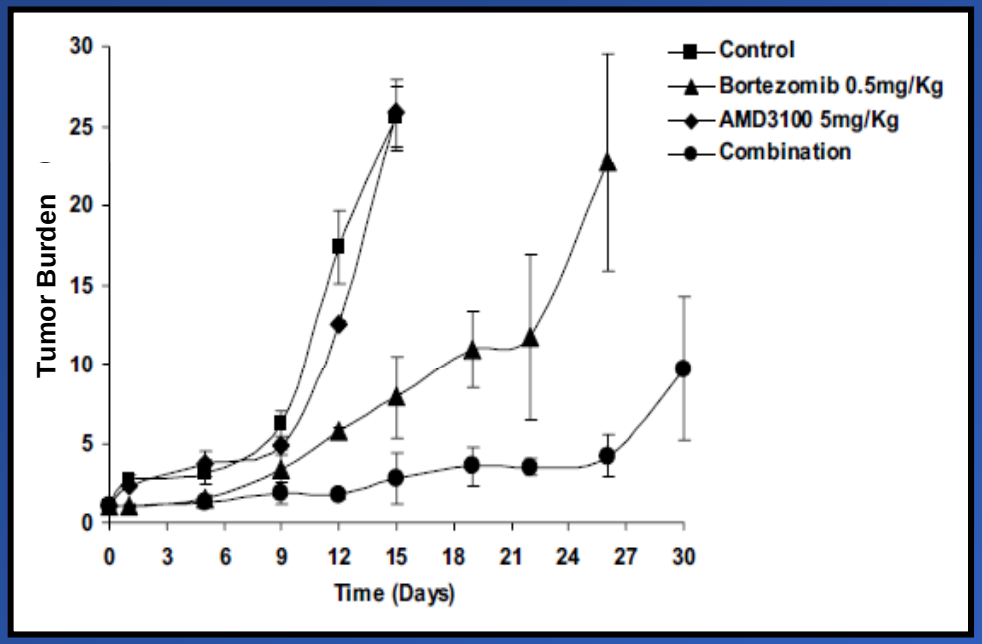
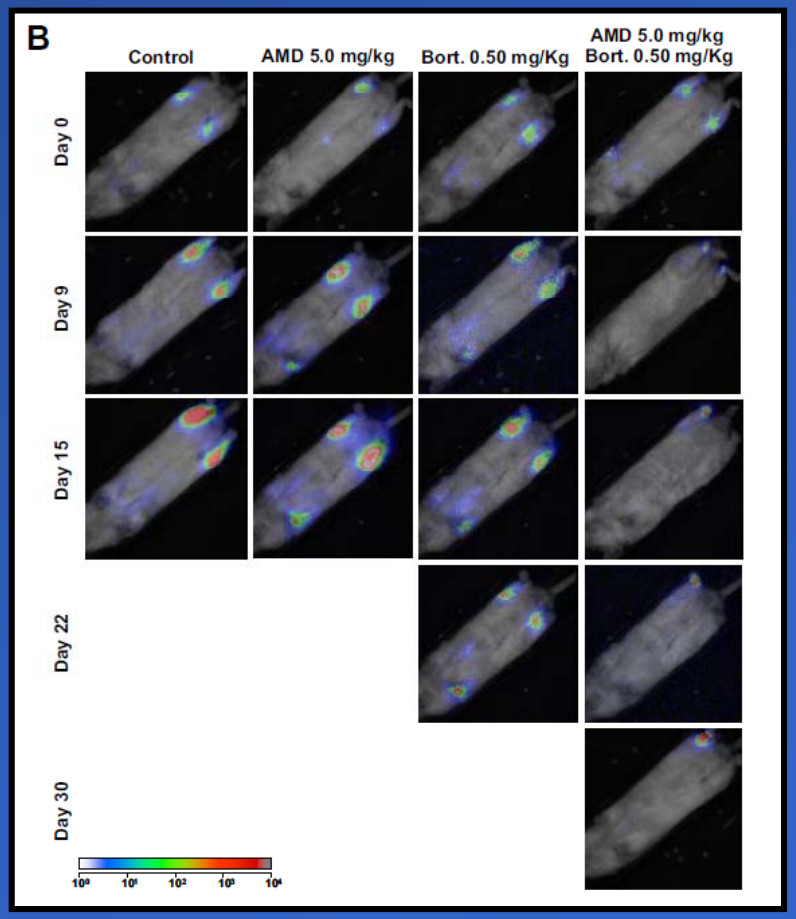
Mice treated with AMD3100 + Bortezomib combination therapy survive significantly longer than mice with Bortezomib alone.





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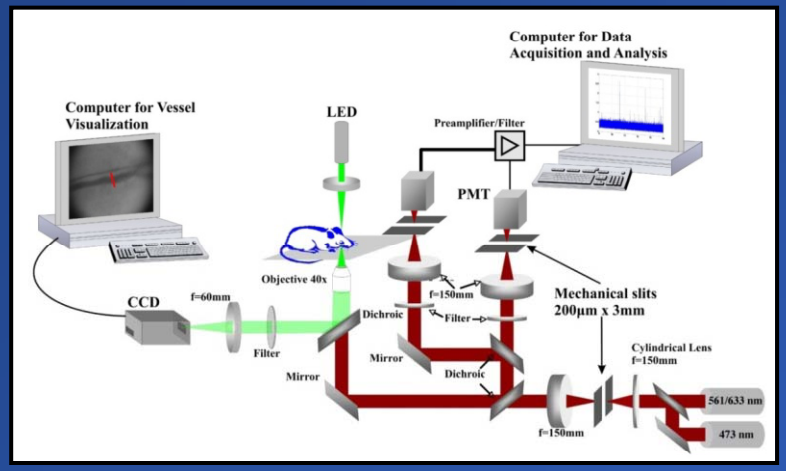
CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy

BLOOD, 30 APRIL 2009 • VOLUME 113, NUMBER 18

Limitation: IVFC of Rare Circulating Cells

The *practical* IVFC detection limit is about 10^3 cells per mL in PB:



~ 1 µL/min peripheral blood sampled

This is insufficient for many applications involving very rare circulating cells, e.g. CTC dissemination during metastasis

< 100 cells/mL PB

Approach #1:
"Computer Vision In Vivo Flow Cytometry"

A Computer Vision Approach to IVFC

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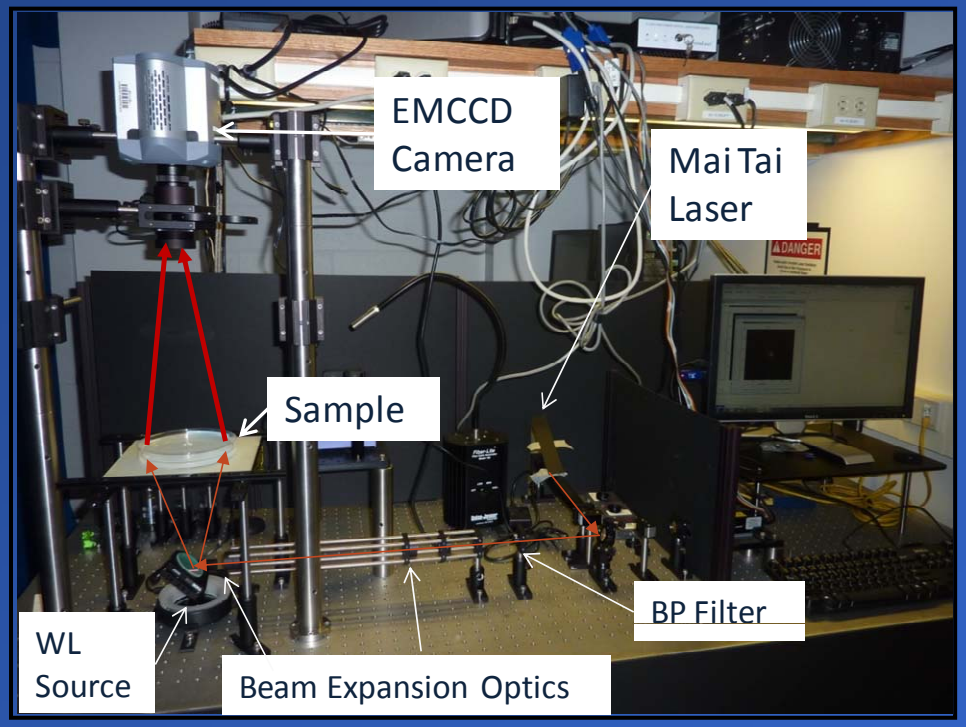
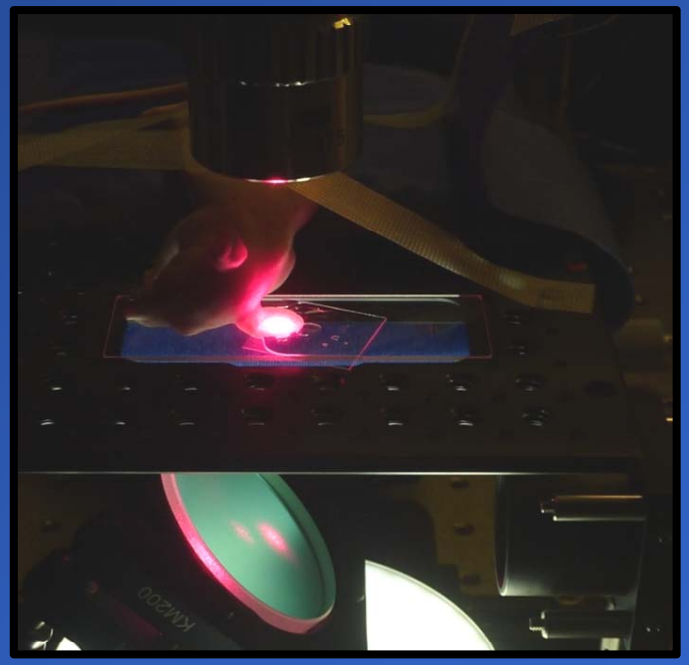
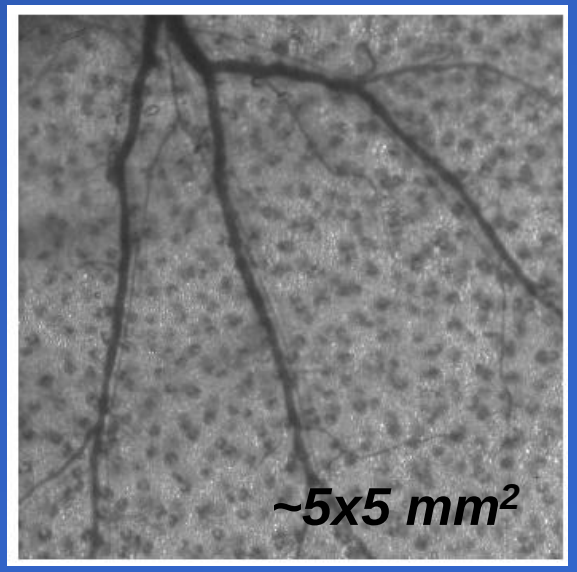
- Transmission fluorescence imaging of ~5 x 5 mm^2 section of mouse ear vasculature
- 20 Hz frame rate

~15 µL of blood flow per minute







Computer Vision In Vivo Flow Cytometry

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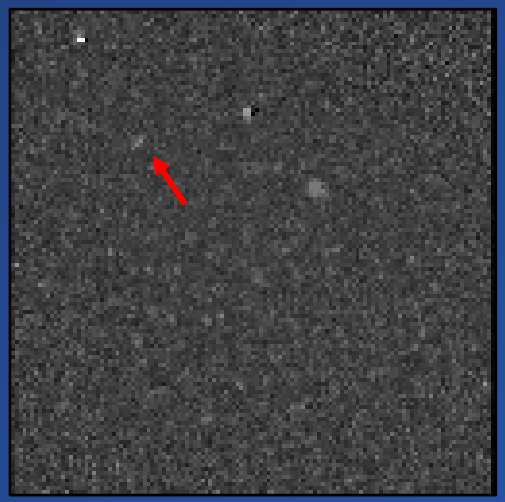
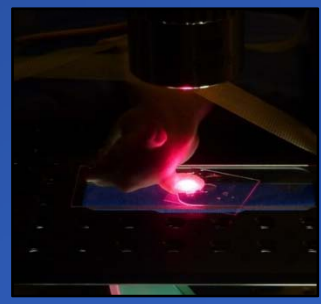
10^3 Vybrant-DiD Labeled Multiple Myeloma Cells *i.v.*



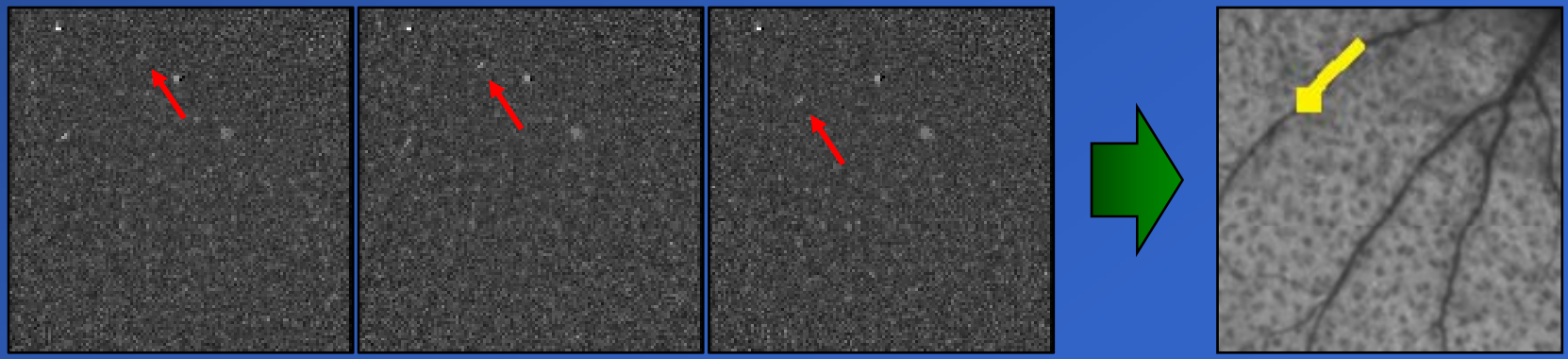


Poor contrast: cell size and intensity is similar to background

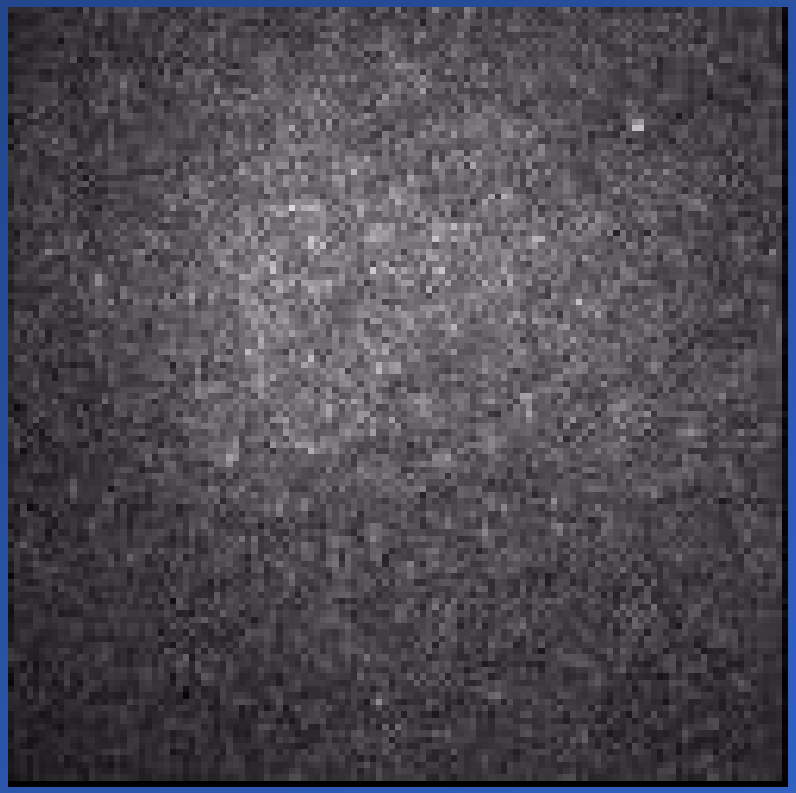
large area, autofluorescence, CCD gain, laser power etc.

Extremely hard to identify circulating cell(s) in single fluorescence images

BUT… circulating cells appear in multiple, temporally related image frames in an image sequence



Circulating Cells – In Vivo Sequence



Want to automate detection and counting!

(only ~ 1-2 cells / minute)

CV-IVFC Algorithm

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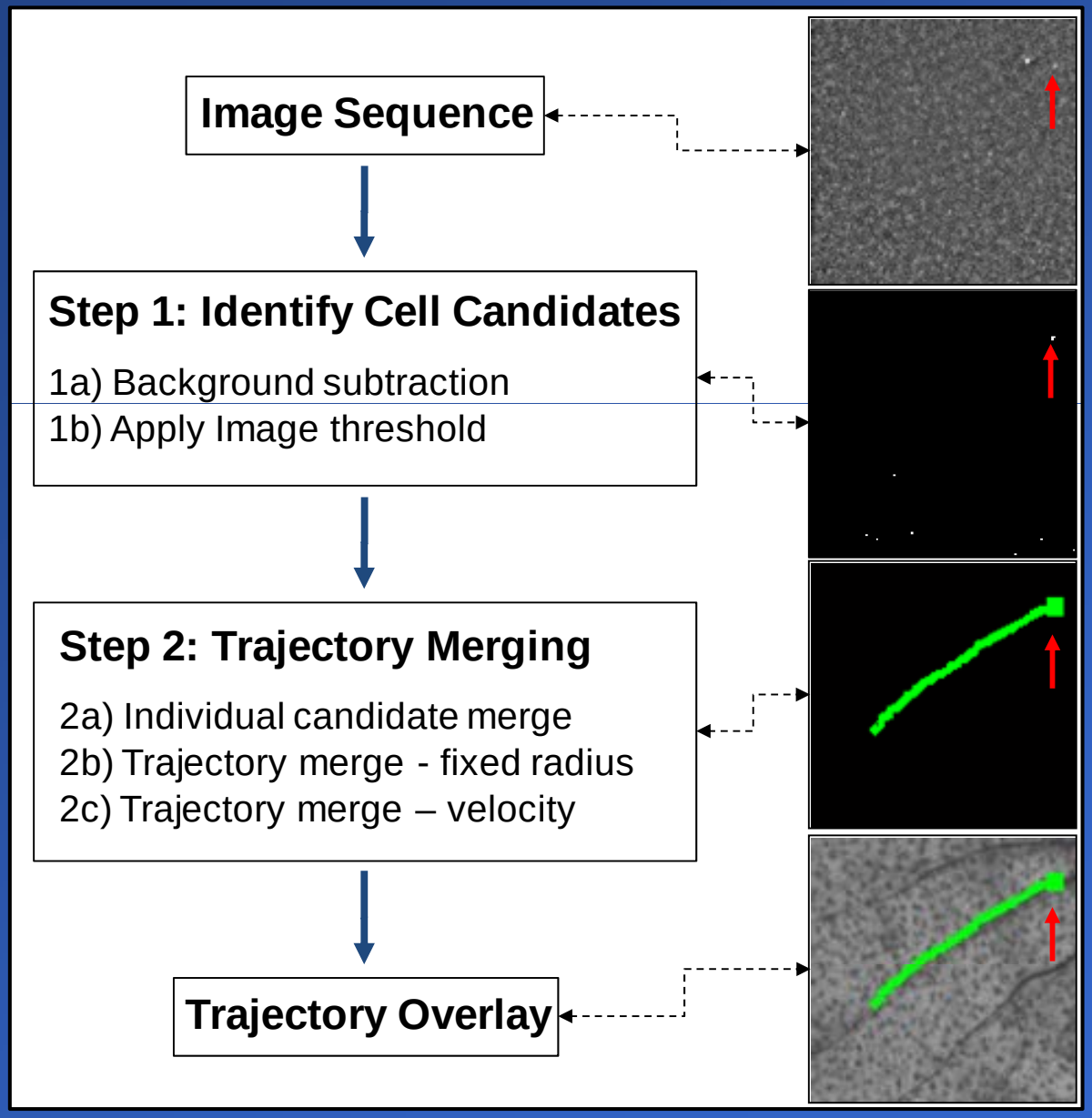

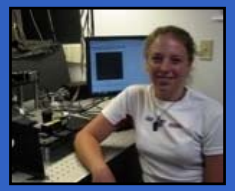

Stacey Markovic
PhD Candidate
ECE

Prof. Octavia Camps -ECE
Computer Vision

Example Cell Tracking Sequences

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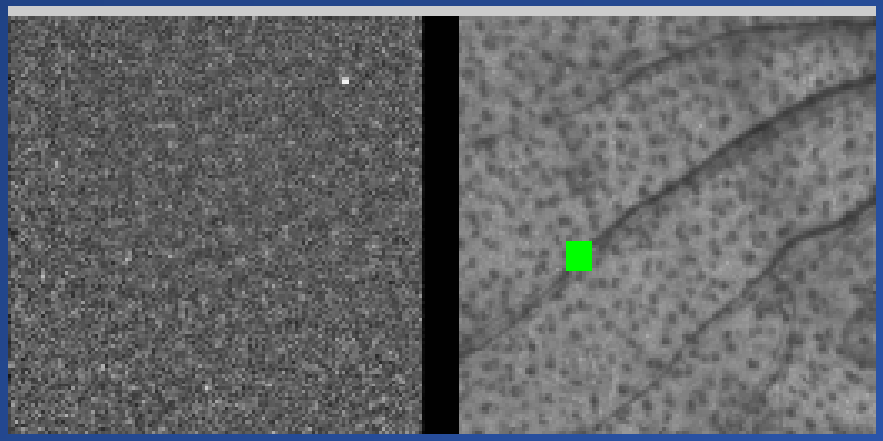
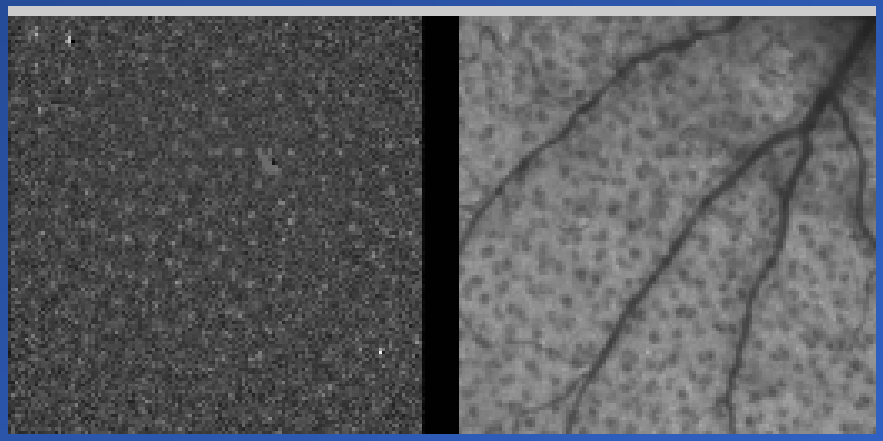
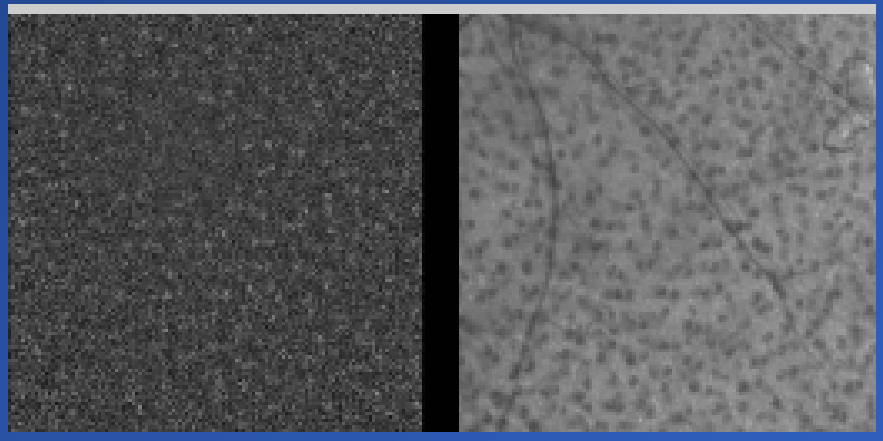
Track 1



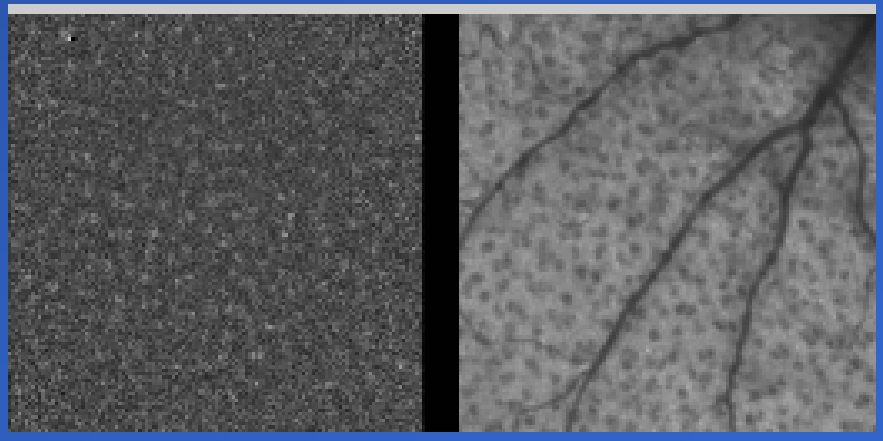
Track 2



Track 3



Track 4



"A Computer Vision Approach to Rare Cell In Vivo Flow Cytometry," Cytometry-A , 2013, 83:1113-23

Computer Vision In Vivo Flow Cytometry

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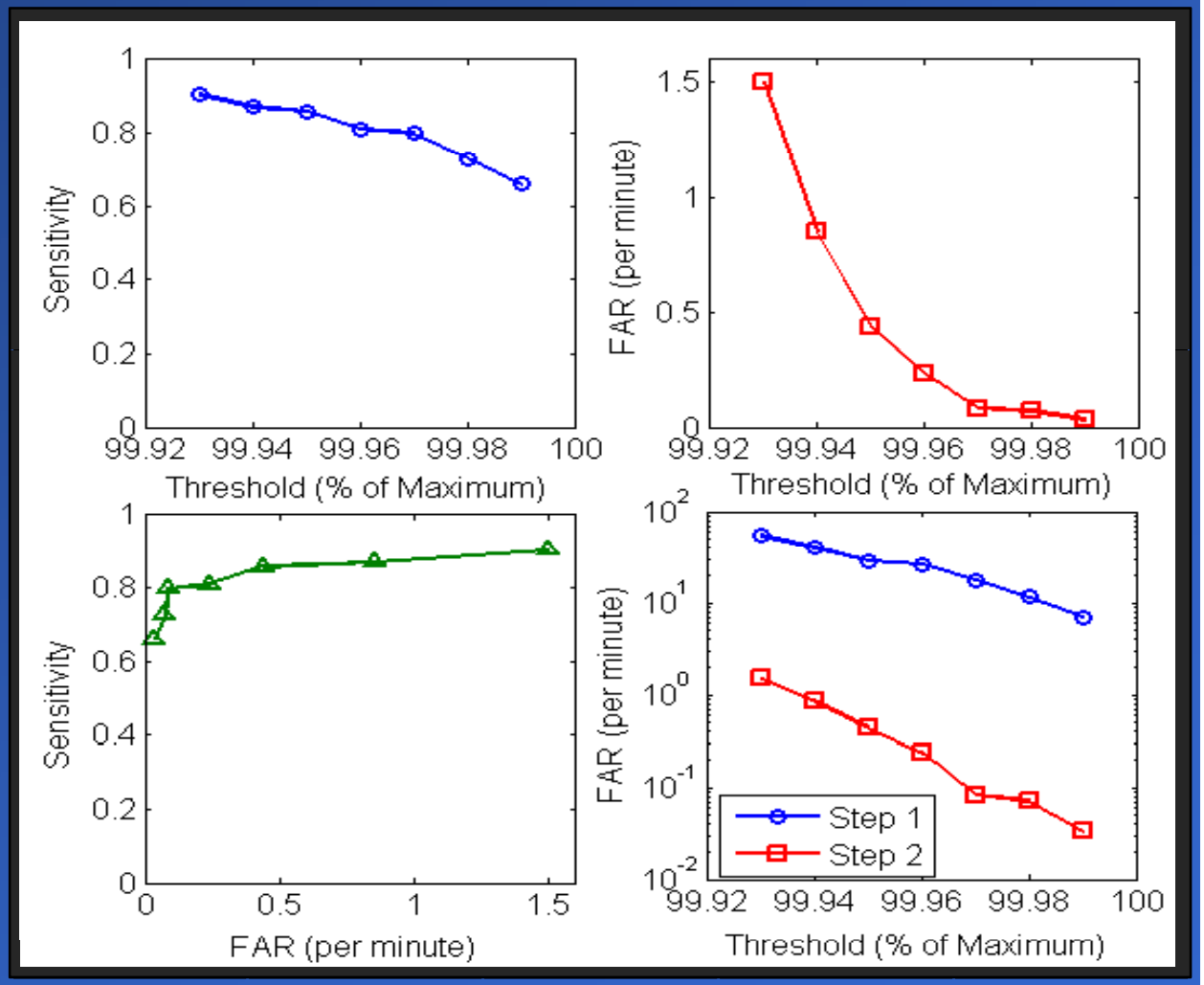
Performance analysis:

- Overall better than 10 cells/mL detection sensitivity

~0.04 false alarms / minute*



Approach #2:
"Diffuse Fluorescence Flow Cytometry"

Diffuse Fluorescence Flow Cytometry

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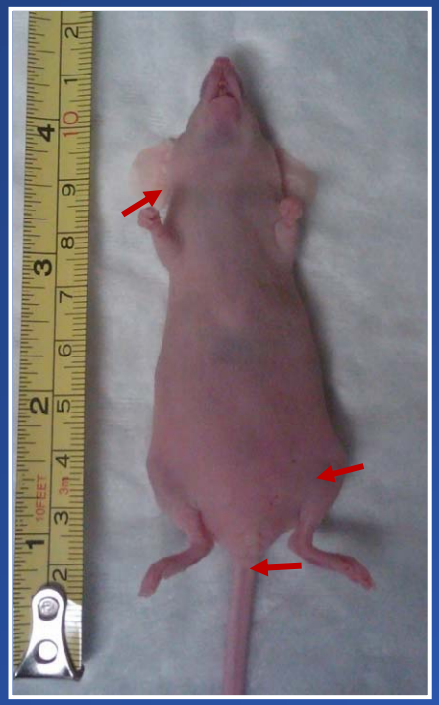
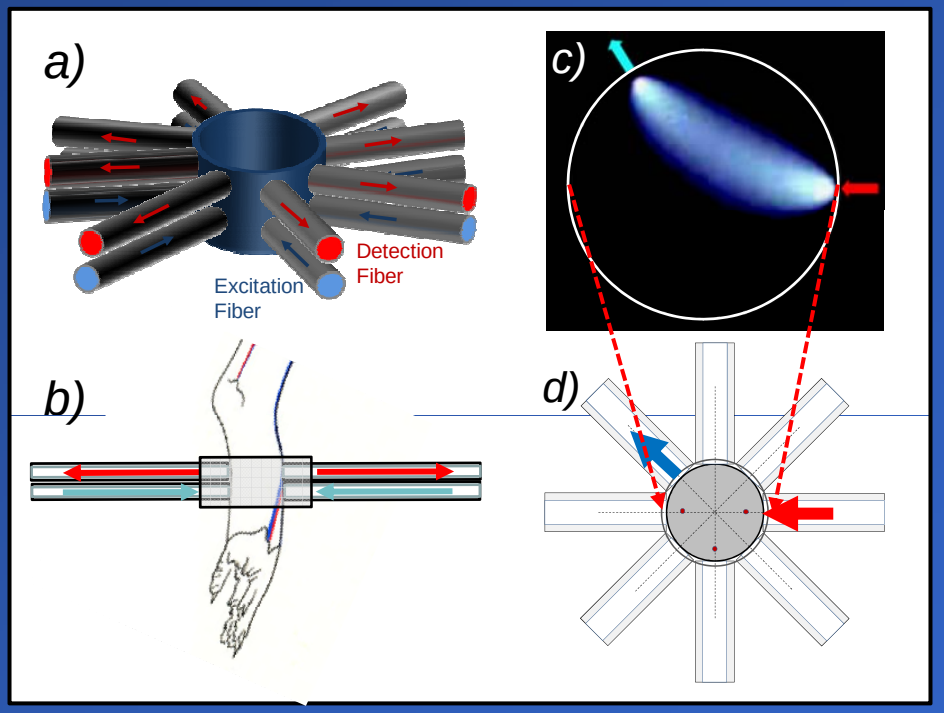
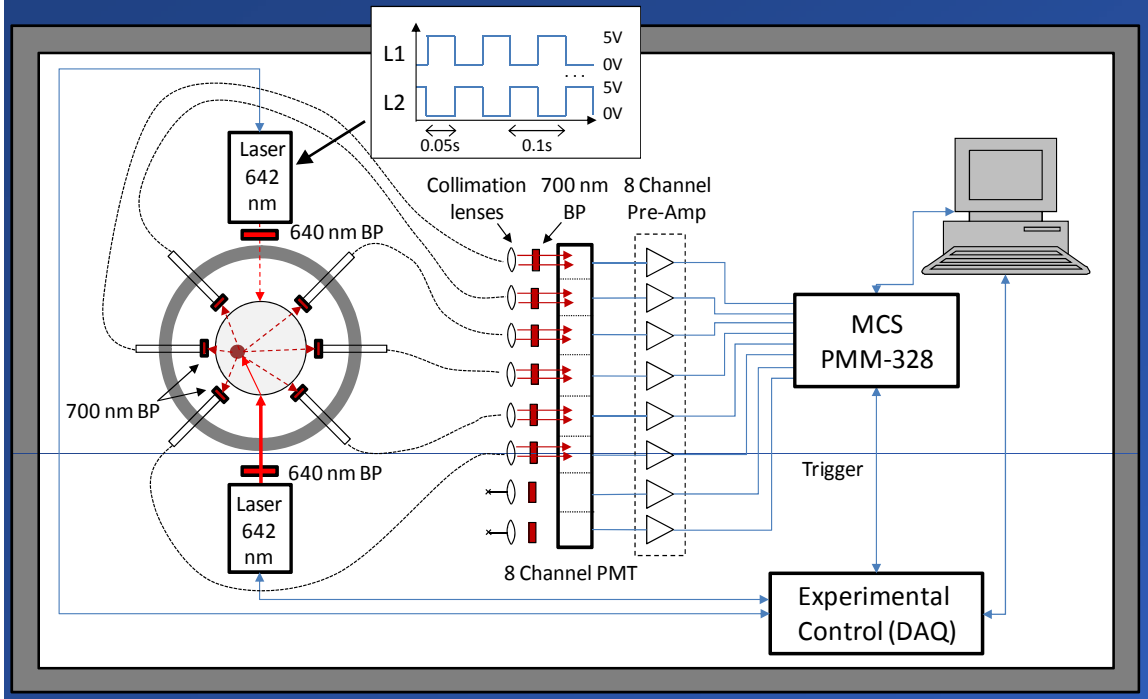


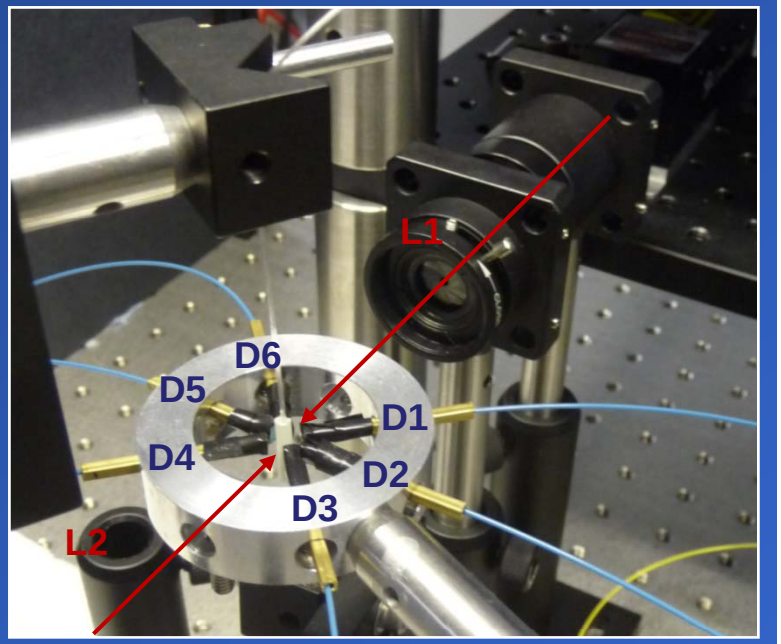


- Limbs, tail are ~2-3 mm in diameter
- 0.2-0.5 mL of blood flow per minute!
  - *whole blood volume (~2 mL) can be sampled in minutes…*

Target *in vivo* sensitivity is <10 circulating cells / mL

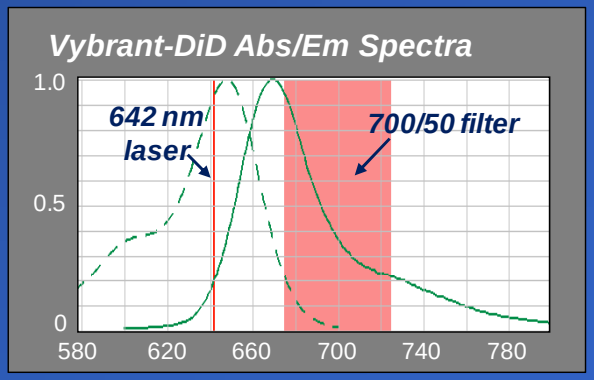
Instrument Design

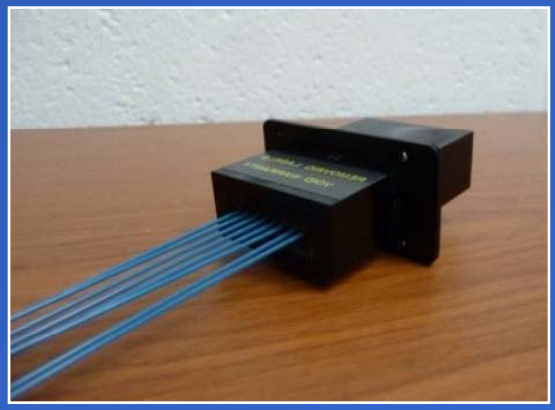




Laser-Filter combination for:

Cy5.5
Alexafluor-680
Vybrant-DiD





8-channel fiber coupled PMT

In Vitro Testing – Calibration Microspheres

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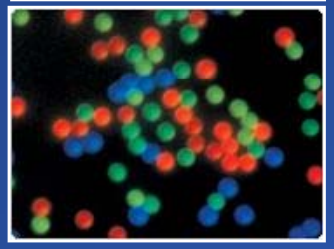
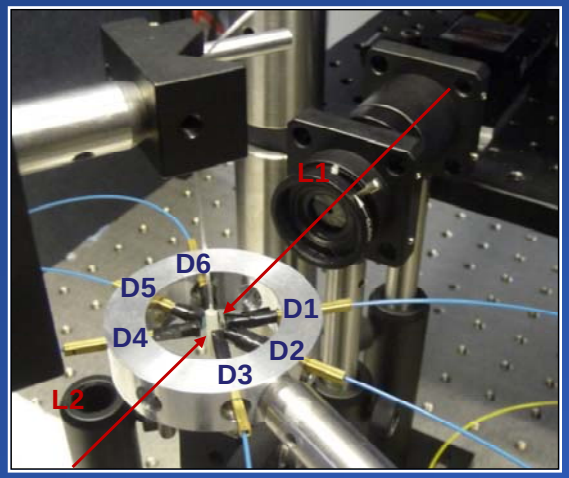
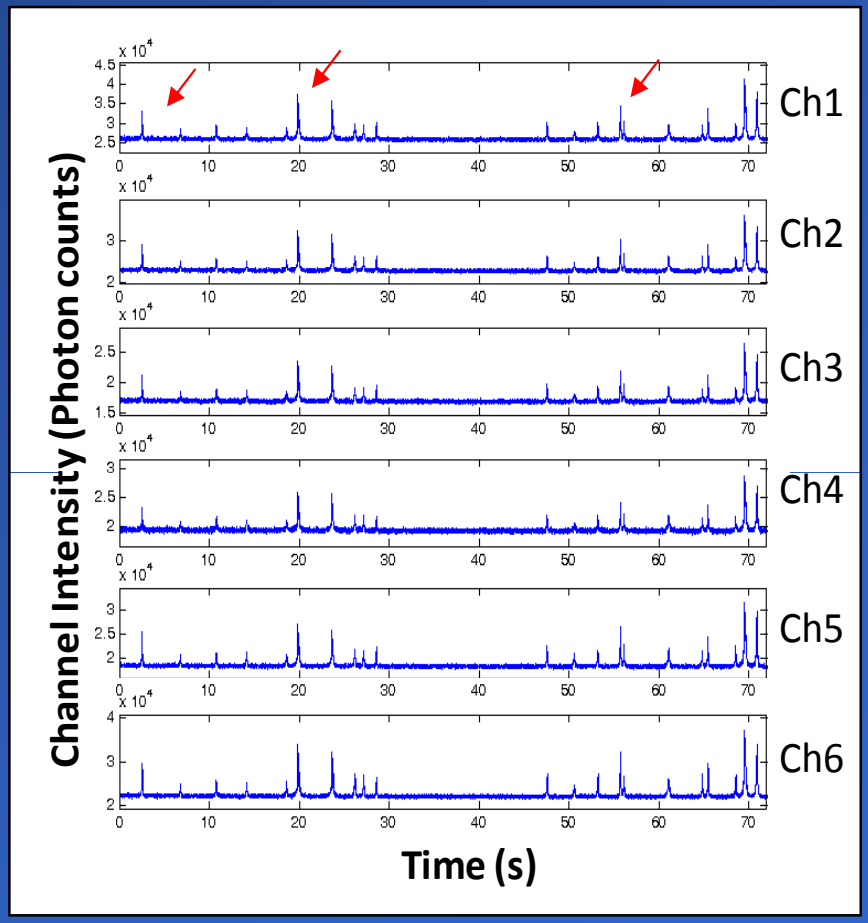
2 x 10^3 microspheres / mL in PBS
2 mm / s linear flow speed







Characterization summary:
Count error less than 10% (compared to commercial flow cytometer)
… in 100-5000 spheres / mL concentration range
… with phantom μ_a from 0.15 to 0.7 cm^{-1}
… with fluorescently-labeled cells as well as microspheres

In Vivo Feasibility Test

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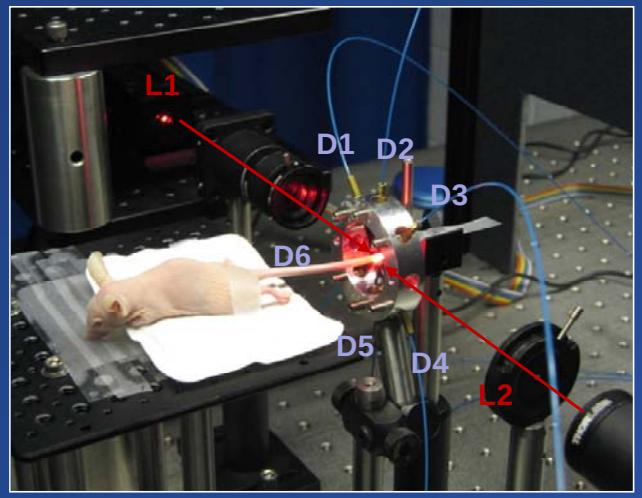
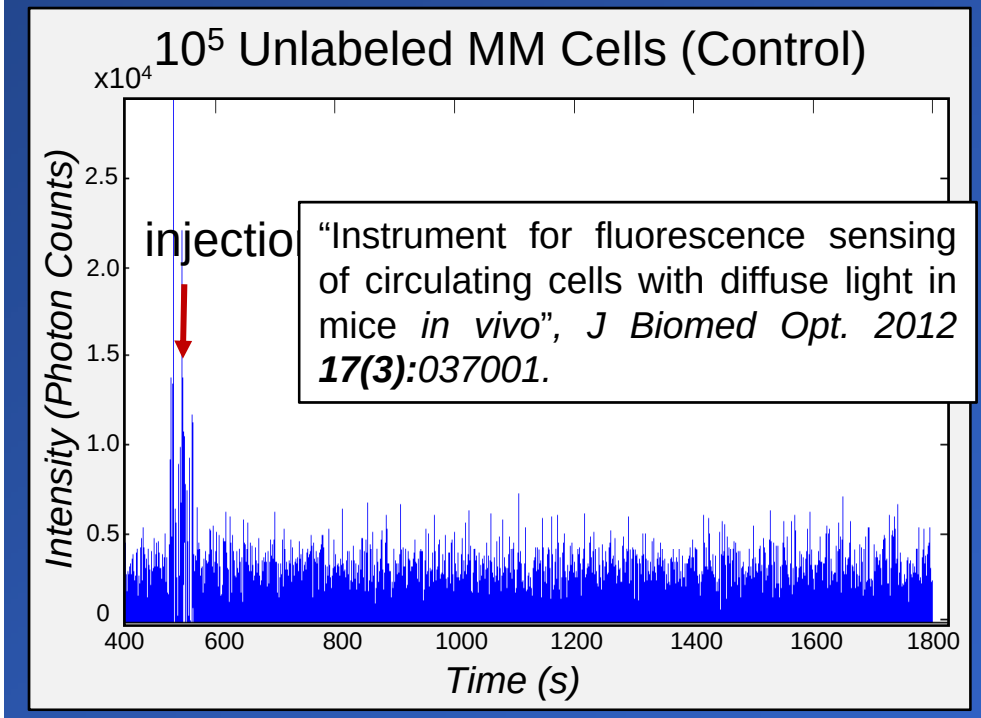
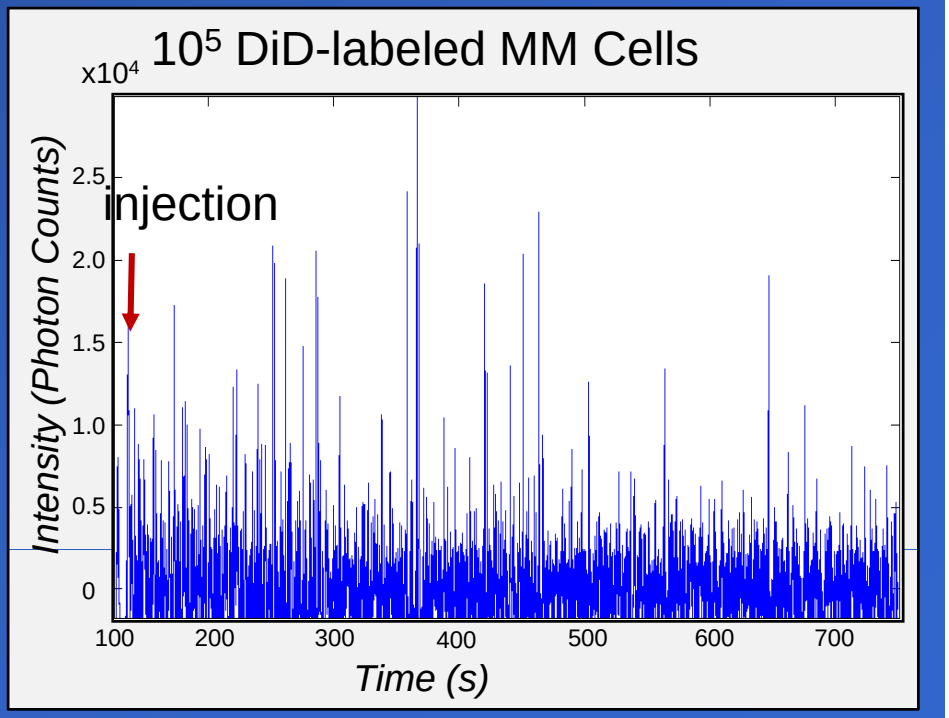


- Retro-orbital injection with **10^5 Multiple Myeloma** Cells – measured through tail
- Vybrant-DiD labeled and unlabeled controls
- Experimental validation of concept





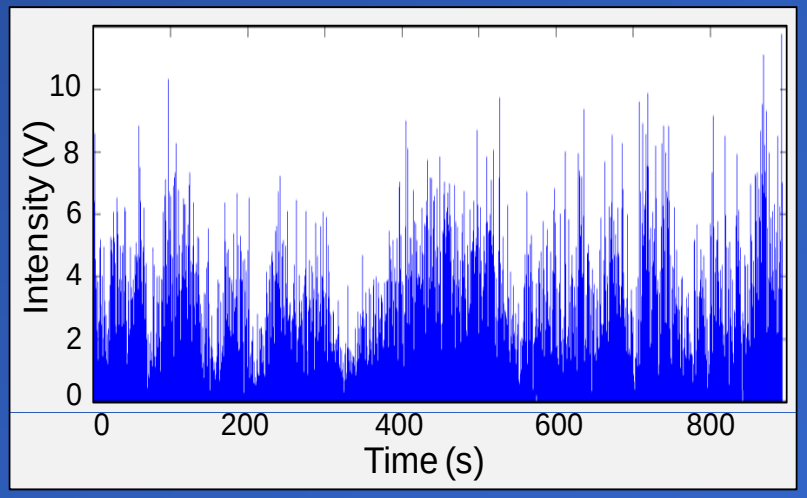
Dual Wavelength Detection

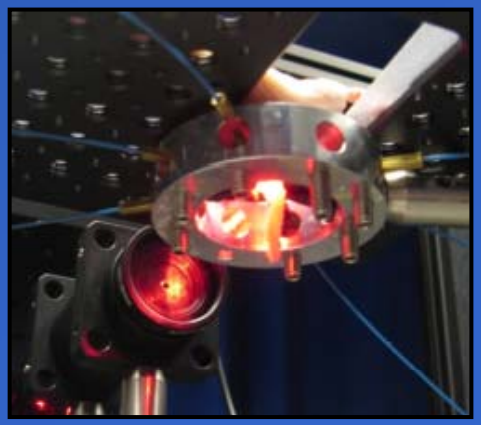
(Movement Artifact Correction)

Movement Artifacts in Hind-leg

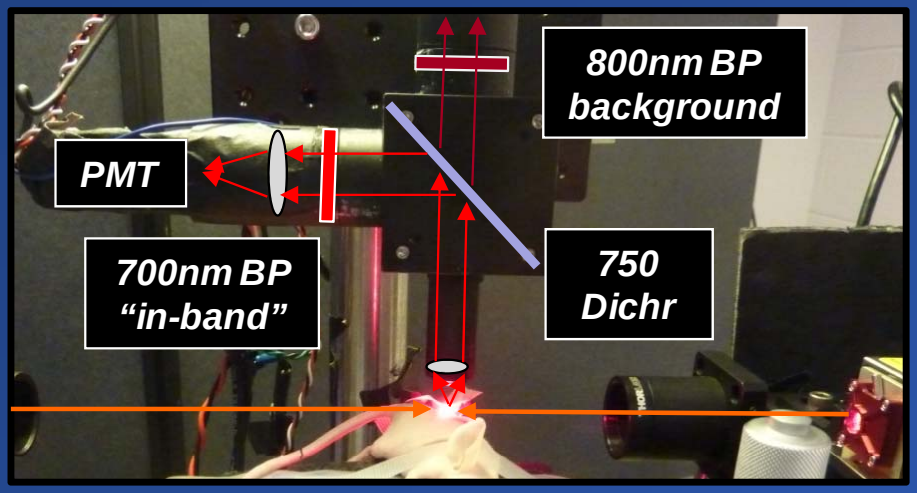
- Tail is highly attenuating (dense connective tissue), which limits DFFC sensitivity
- Hind-leg has much less attenuation
- But… significant movement artifacts observed in properly anesthetized control mice

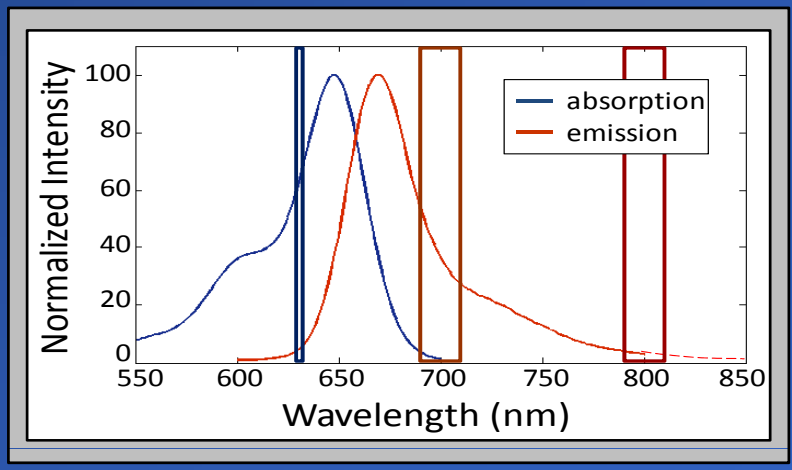
Example 15 minute trace, control mouse hind-leg:



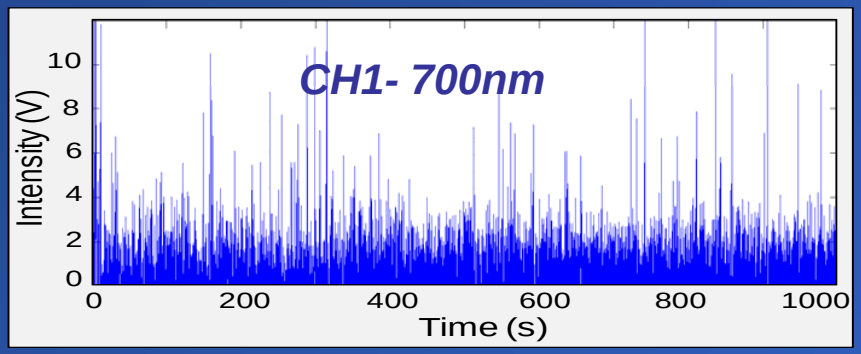


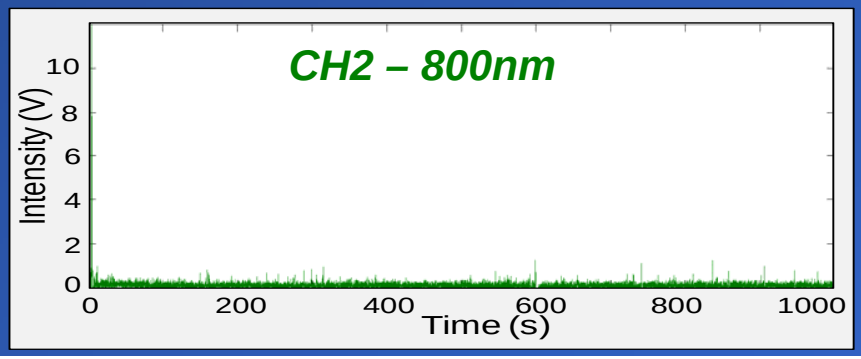
Dual Wavelength Detection



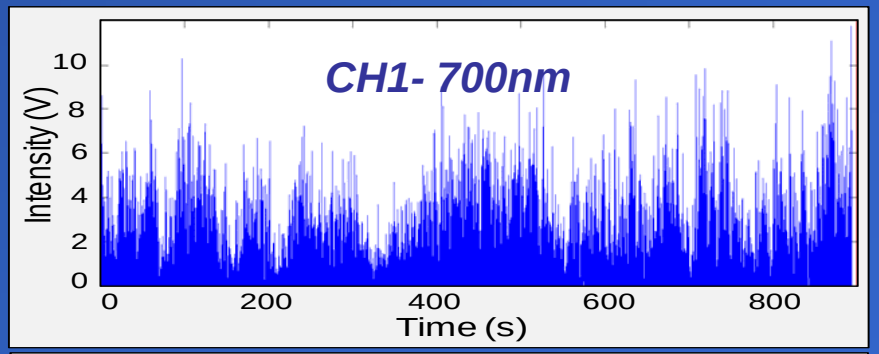


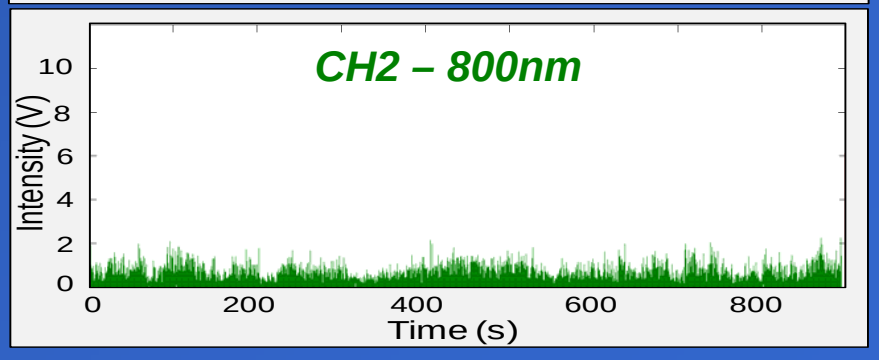
10^4 Mesenchymal Stem Cells i.v.





Control Mice





Motion Artifact Removal

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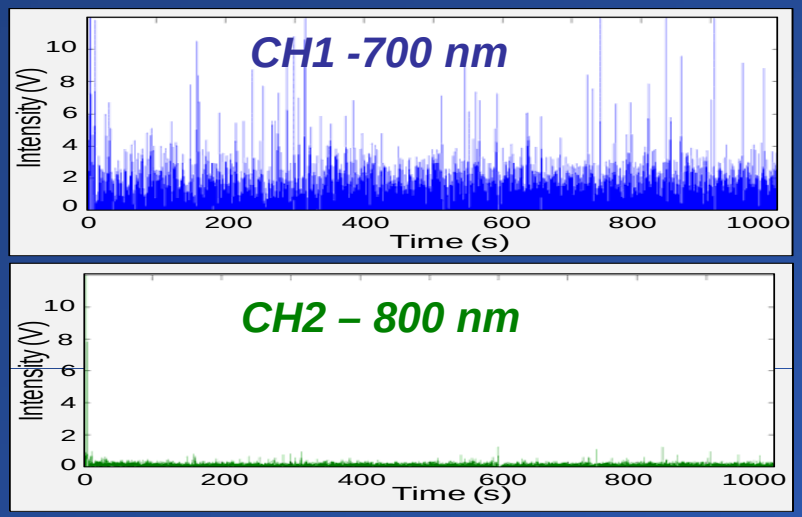
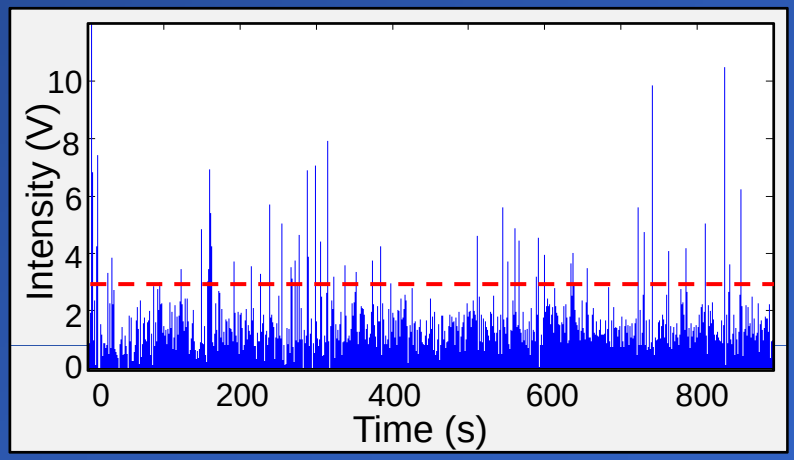
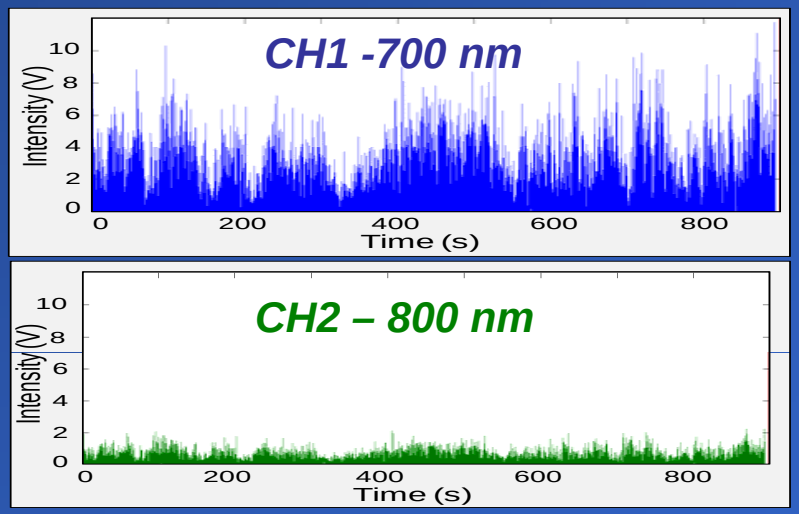
MSC Injected Mice (~5 x 10^3/mL)

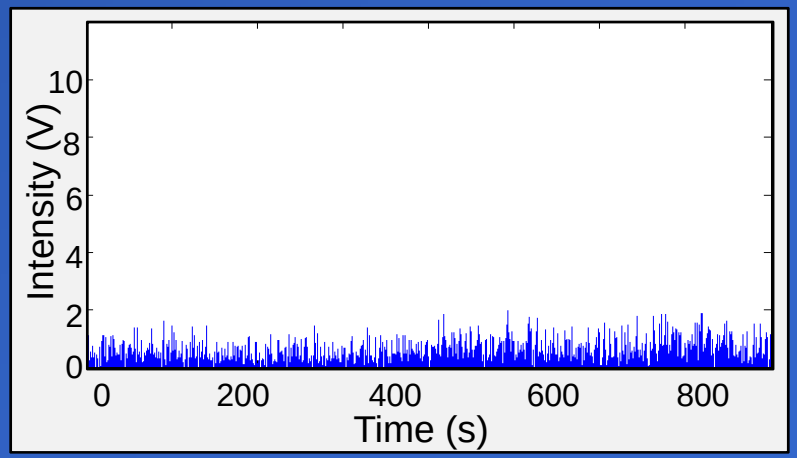




Possible to count cells at this concentration…

Control Mice





Quantitative Accuracy - Clearance Kinetics

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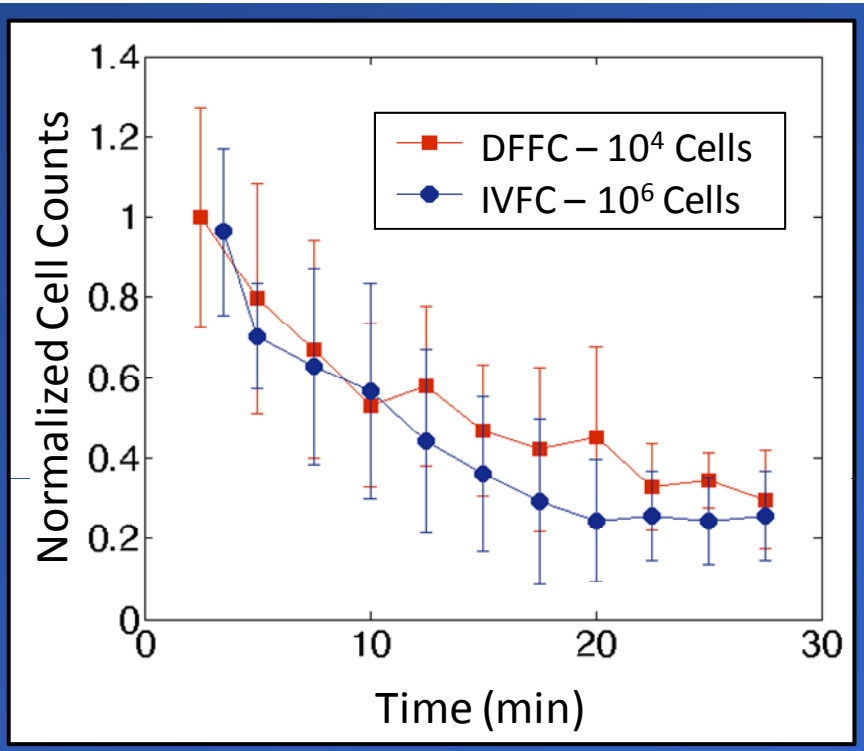
Mesenchymal Stem Cells, DiD labeled. 5 x 10^3 cells / mL injected

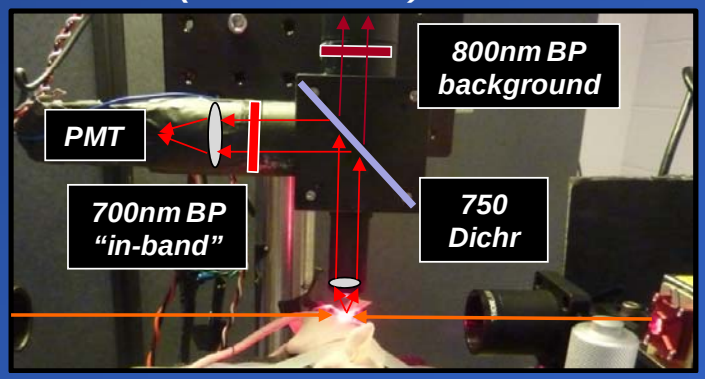
Compared MSC cell clearance kinetics measured with DFFC compared to "gold standard" IVFC

DFFC sensitivity in 15 min acquisition

~ 1 cell / mL

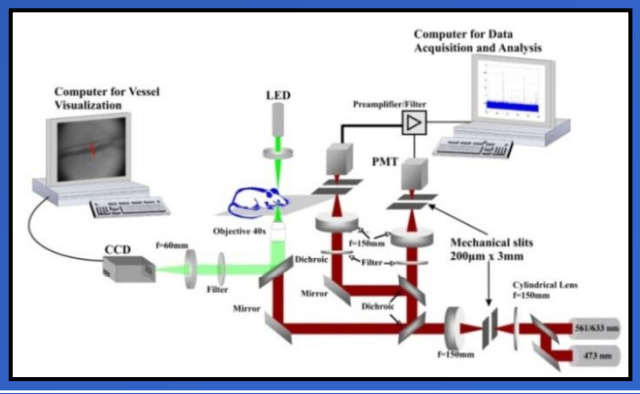


DFFC (Version 2)*



VS.

IVFC



*"Improved Diffuse Fluorescence Flow Cytometer Prototype for High Sensitivity Detection of Rare Circulating Cells *In Vivo*" *Journal of Biomedical Optics, 2013, 18:077002*

Tomography

Tomography: Motivation and Approach

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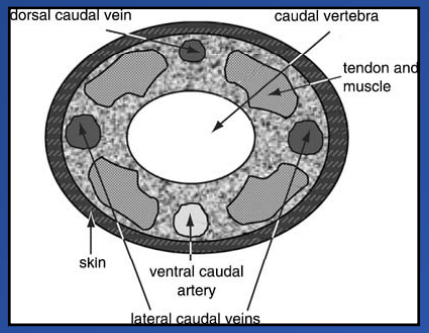
Motivation: Multiple blood vessels in the field of view can lead to over-counting errors (i.e. exit and return)

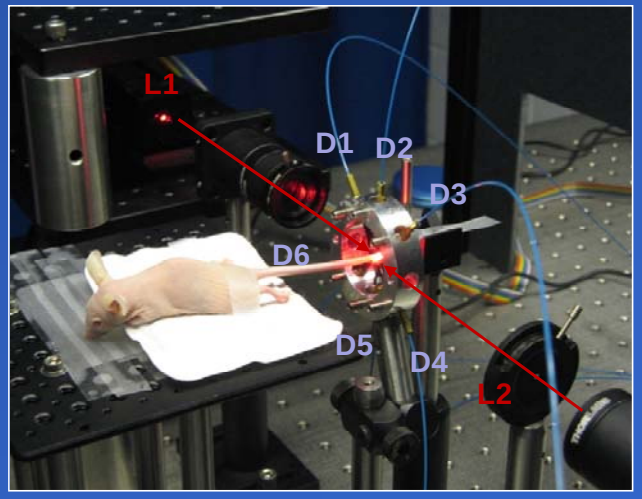


Would like spatial information to correct for this and obtain,

e.g. counts / blood vessel

Our instrument generates
6 detectors x 2 sources

= 12 measurements at 10Hz



This is a sparse diffuse fluorescence tomography data set

Tomography – Phantom Testing

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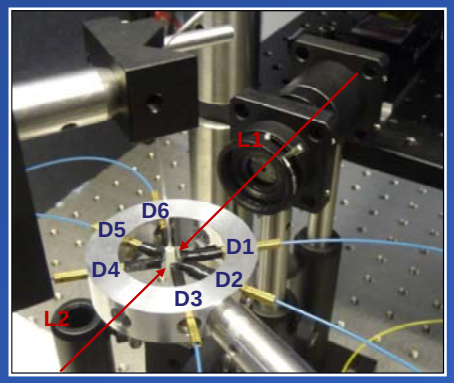
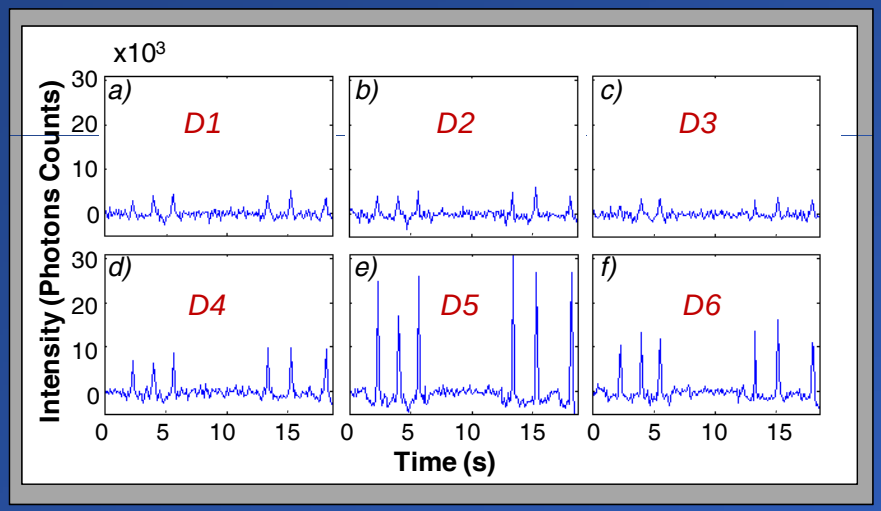
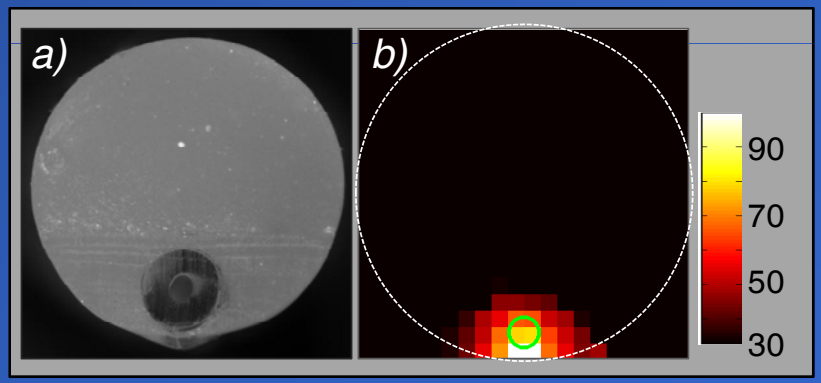
Generally, it works fairly well (~500µm)

Robustly reconstructs correct clock position
…but, depth is poorly resolved*

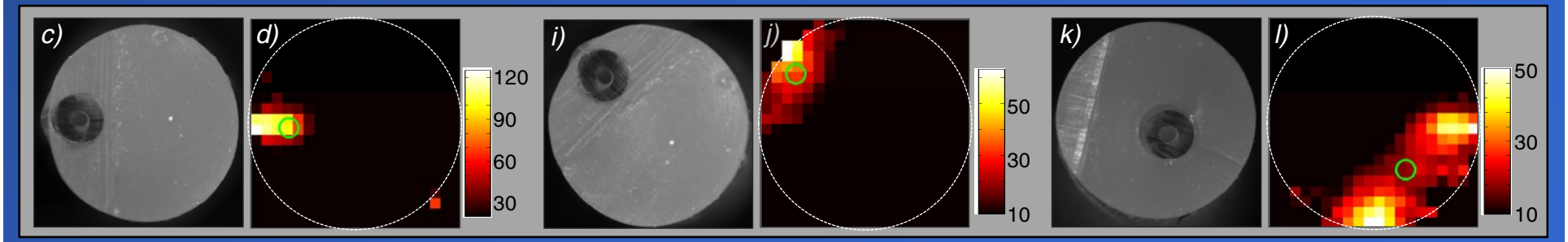






Reconstruction details: 250µm grid, r-ART inversion, 25 iterations

Other orientations:



In Vivo Feasibility Tests

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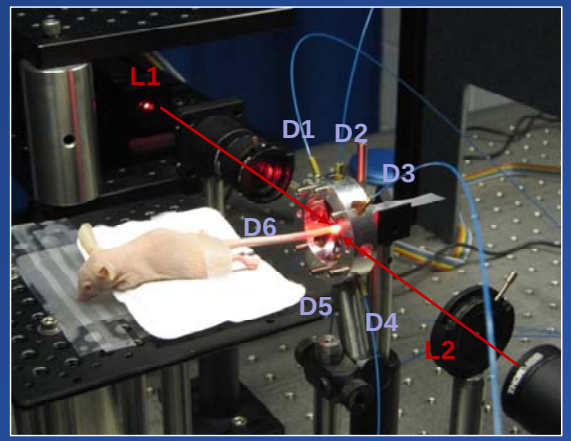
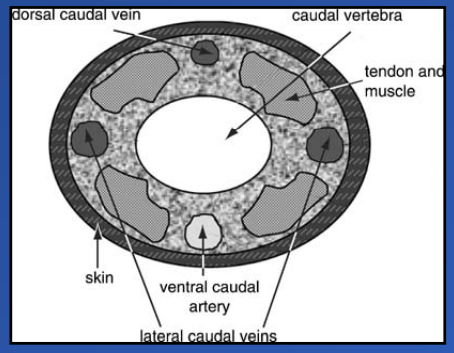
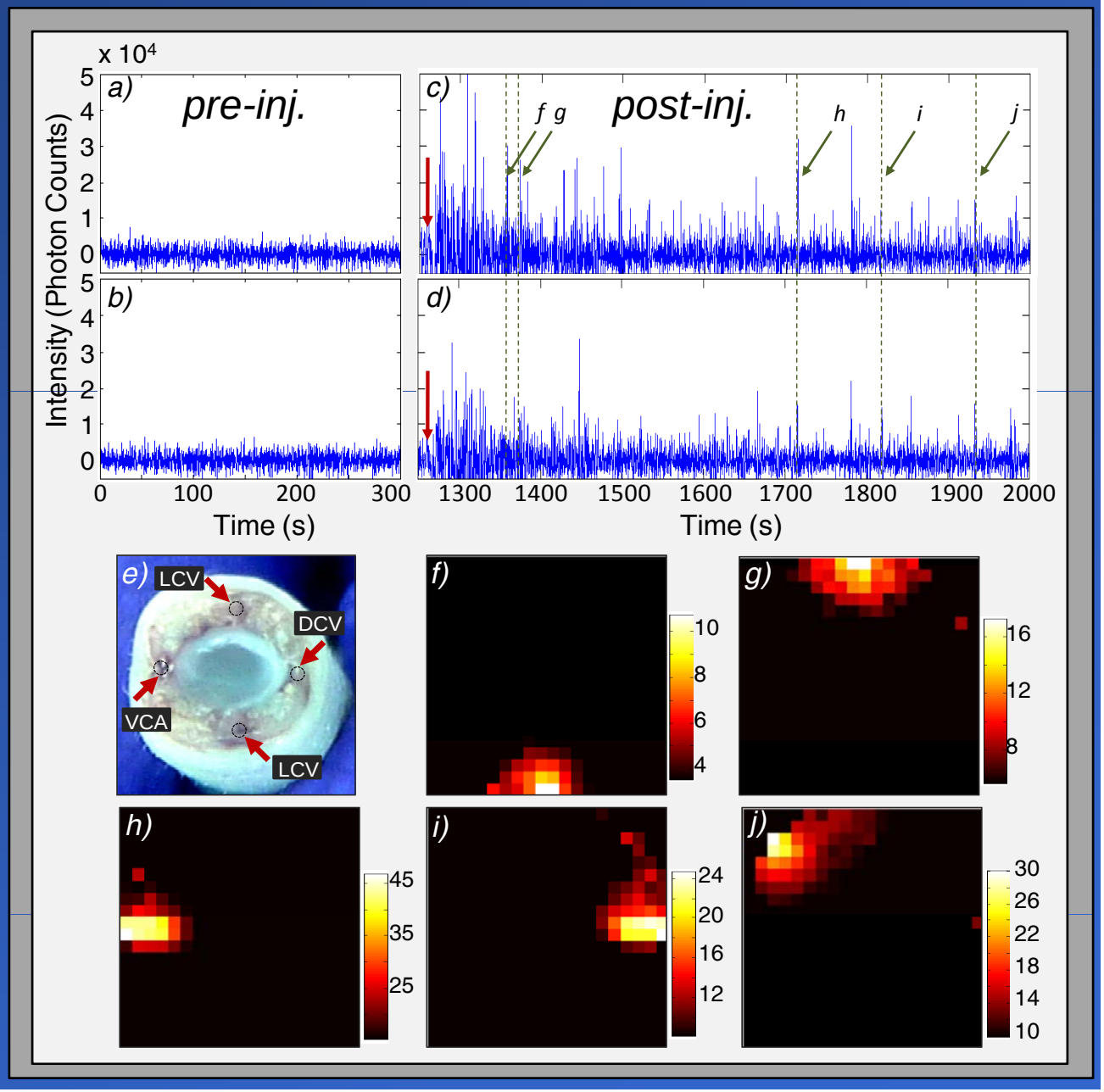
10^5 Multiple Myeloma
cells injected retro-orbitally in nude mice







"Tomographic sensing and localization of fluorescently labeled circulating cells in mice *in vivo*.", *Phys Med. Biol. 2012* ***57(14)***:4627-4641.

Sparsity as an Imaging Prior

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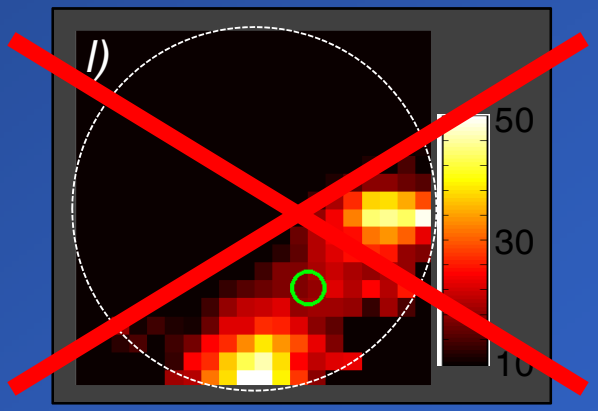
We know *a priori* that the solution should be very sparse (single cell)

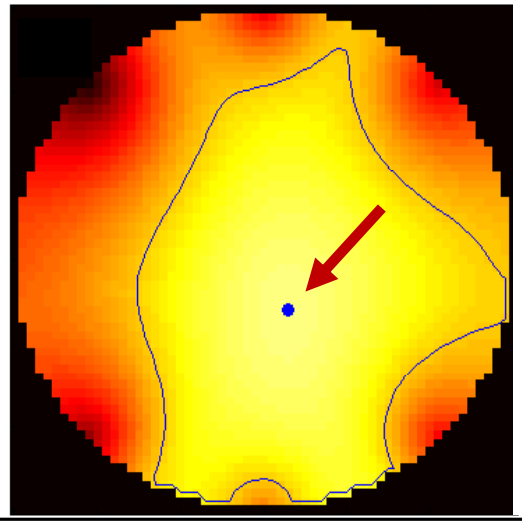
i.e. size of one cell < one pixel

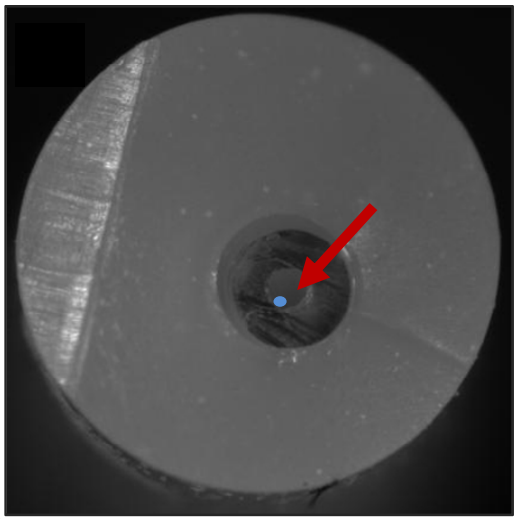


- Example - Maximum likelihood estimation method for *point* targets:

Maximum Likelihood (Point target)

ML Overlay





"Maximum likelihood reconstruction of extremely sparse solutions in diffuse fluorescence flow cytometry", Optics Letters, 2013, 38: 2357-2359

Applications:

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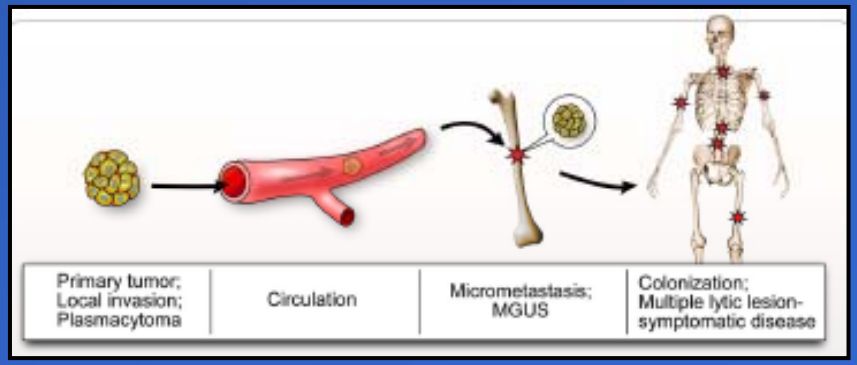
We have reached target sensitivity range of <100 cells/mL …

Multiple Myeloma:

- Study of mobilization of Minimal Residual Disease (MRD) in vivo
- Study of mobilization of sub-populations of MM cells
- Study of *early-stage* MM dissemination via circulatory system

Circulating Tumor Cells and Metastasis:

- Measurement of onset of CTCs in a spontaneous tumor model
- Testing of novel drugs for early-stage metastasis



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Markovic, S., et. al. (2013) “A computer vision approach to rare-cell in vivo fluorescence flow cytometry,” *Cytometry A*, 83(12):1113-1123.

Pera, V., et. al. (2013) “Maximum likelihood tomographic reconstruction of extremely sparse solutions in diffuse fluorescence flow cytometry,” *Optics Letters*, 38(13): 2357-2359.

Pestana, N., et. al. (2013) “Improved diffuse fluorescence flow cytometer prototype for high sensitivity detection of rare circulating cells in vivo,” *Journal of Biomedical Optics*, 18(7): 77002.

Zettergren E., et. al. “Tomographic sensing and localization of fluorescently labeled circulating cells in mice in vivo,” *Physics in Medicine and Biology*, 57(14): 4627-4641.

Zettergren, E., et. al. (2012) “An instrument for fluorescence sensing of circulating cells with diffuse light in mice in vivo,” *Journal of Biomedical Optics*, 17(3): 037001.

Zettergren, E.W., et. al. (2011) “Validation of a device for fluorescence sensing of rare circulating cells with diffusive light in an optical flow phantom model,” *Conf. Proc IEEE Eng Med Biol Soc*. 486-489.

Zettergren, E.W., et. al. (2011) “Sensing and enumerating rare circulating cells with diffuse light” *Proceedings of the SPIE Photonics West, BiOS*, 7902: 790229.

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Northeastern University