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 High-Sensitivity IVFC
   "Computer Vision In Vivo Flow Cytometry"
   "Diffuse Fluorescence In Vivo Flow Cytometry"
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...

**Limitations?**
- Frequency of sampling (survival experiments)
- Sensitivity (blood sample volume)
- Storage, handling, enrichment of samples
**In Vivo Flow Cytometry**

Fluorescence microscopy IVFC; mouse ear or retina

*Continuous, non-invasive measurements*

**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.

*C P Lin, I Georgekoudi, VP Zharov, VV Tuchin, TB Norris, X Wei, etc.*

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

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
**Tx Strategy: Keep MM Cells In Circulation**

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

“mobilization therapy”

**AMD3100; CXCR-4 inhibitor**

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

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

AK Azab et. al.

BLOOD, 30 APRIL 2009 • VOLUME 113, NUMBER 18
The *practical* IVFC detection limit is about $10^3$ cells per mL in PB:

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

- Transmission fluorescence imaging of ~5 x 5 mm² section of mouse ear vasculature
- 20 Hz frame rate

~15 µL of blood flow per minute

~5x5 mm²
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**

1. **Step 1: Identify Cell Candidates**
   - 1a) Background subtraction
   - 1b) Apply Image threshold

2. **Step 2: Trajectory Merging**
   - 2a) Individual candidate merge
   - 2b) Trajectory merge - fixed radius
   - 2c) Trajectory merge – velocity

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**Image Sequence**

**Trajectory Overlay**

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**Stacey Markovic**

PhD Candidate

ECE

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**Prof. Octavia Camps**

ECE

Computer Vision
Example Cell Tracking Sequences

Track 1

Track 2

Track 3

Track 4

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

- 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

Vybrant-DiD Abs/Em Spectra

8-channel fiber coupled PMT
**In Vitro Testing – Calibration Microspheres**

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 $\mu_a$ from 0.15 to 0.7 cm$^{-1}$
... with fluorescently-labeled cells as well as microspheres
In Vivo Feasibility Test

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

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**10^5 Unlabeled MM Cells (Control)**

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**10^5 DiD-labeled MM Cells**

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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**

10⁴ Mesenchymal Stem Cells i.v.

**CH1 - 700nm**

**CH2 – 800nm**

Control Mice

**CH1 - 700nm**

**CH2 – 800nm**
Motion Artifact Removal

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

CH1 - 700 nm

CH2 – 800 nm

Possible to count cells at this concentration…

Control Mice

CH1 - 700 nm

CH2 – 800 nm
Mesenchymal Stem Cells, DiD labeled. $5 \times 10^3$ cells / mL injected

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

DFFC sensitivity in 15 min acquisition

$\sim 1$ cell / mL

**DFFC (Version 2*)**

800nm BP background

700nm BP “in-band”

**IVFC**

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"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**

*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

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

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

Sparsity as an Imaging Prior

We know \textit{a priori} that the solution should be very sparse (single cell)

\textit{i.e. size of one cell < one pixel}

- Example - Maximum likelihood estimation method for \textit{point} targets:

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

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


Acknowledgements

Lab Members:

Graduate Students
- Zhi Li
- Binlong Li
- Juhua Huang
- Stacey Markovic
- Ying Mu
- Vivian Pera
- Noah Pestana
- Niksa Valim
- Eric Zettergren

Undergraduate Students
- Ryan Duoress
- Oliver Li
- Timothy Rossini
- Tushar Swamy
- Kristin Solomon

Collaborators:

Harvard-MGH
- Prof. Charles P. Lin
- Prof. Bakhos Tannous

Harvard-DFCI
- Prof. Irene Ghobrial

Northeastern
- Prof. Shashi Murthy
- Prof. Octavia Camps
- Prof. Dana Brooks

Support:

NIH-NHLBI - R21HL098750 (Niedre)
NIH-NIBIB - R01 EB012117 (Niedre)
Massachusetts Life Sciences Center
New Investigator Award (Niedre)
Northeastern University