Fit-for-purpose considerations for cell counting using image cytometry

April 10, 2017
Cell Counting Workshop

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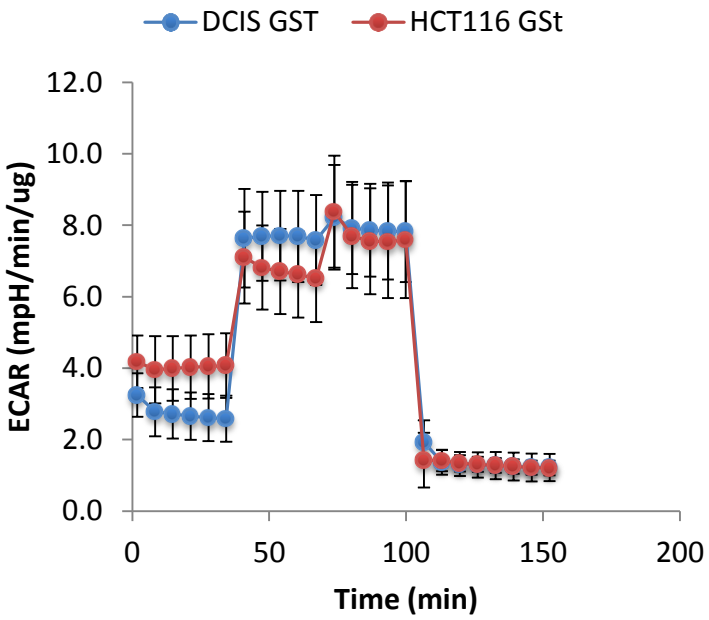
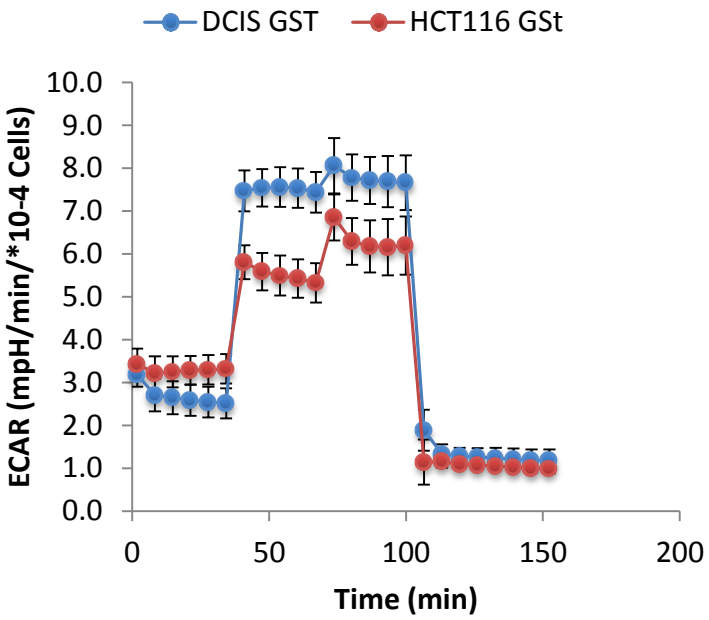
Nexcelom Bioscience

Does cell counting produce better results?

Glycolytic function measured using Seahorse

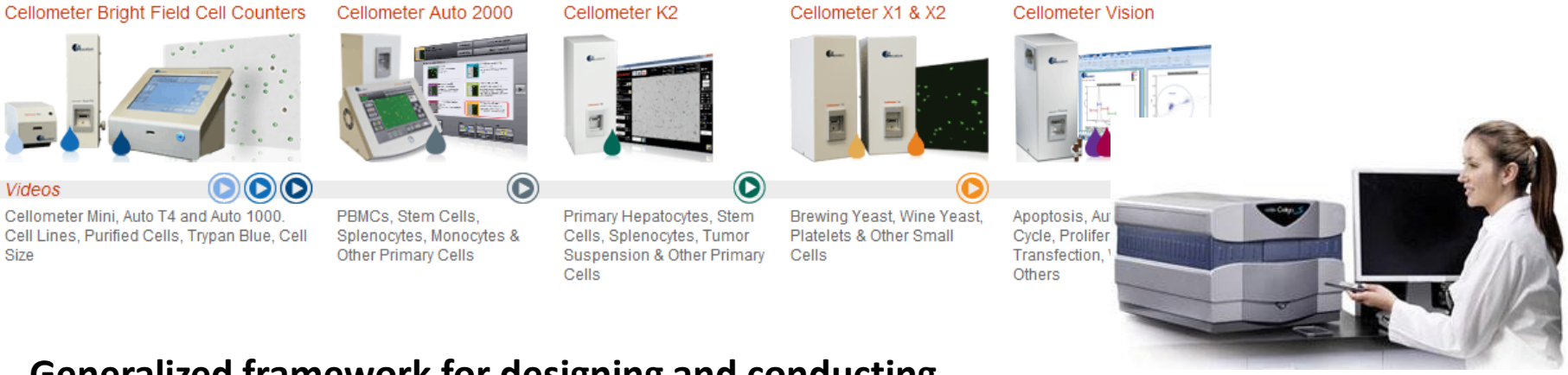


Normalization using protein quantity



Normalization using cell count

Which cell counter is right for me?
It depends!



Generalized framework for designing and conducting cell measurements

- Well defined quality attribute
- **Well-designed measurement that are fit-for-purpose**
- Robust measurement with built-in measurement assurance
- Appropriate documentation, reporting, and communication

Lin-Gibson et al., Cytotherapy. 2016 Oct; 18 (10):1241-4

Fit-for-purpose investigation is the most important work prior to verification and validation.

Three key fit-for-purpose considerations for cell counting

1. Understand cell preparation
   - What types of cells are in the cell preparation?
2. Evaluate cell counting assays for selectivity for the desired cell type
   - How do each cell counting assay work? Does it have the selectivity to count desired cell type within this cell preparation?
3. Determine tolerance in sample processing prior to cell counting
   - How much processing steps are acceptable before cell counting?

Three use cases to illustrate these key considerations for fit-for-purpose

- Case I: count cells for large tumor cell line panels used for oncology drug screening
- Case II: count cells for HPC based cellular processing
- Case III: count cells for mouse experiments

A few definitions

A laboratory conducts multiple biological operations with varied cell counting needs

- Biological operation
  - A series of experiments to produce results
- Cell preparation
  - Cell sample to be analyzed for cell count
- Cell counting assay
  - The specific measurement to obtain cell counts
- Cell counting system
  - Hardware, software, reagents to produce cell counting results

Understand cell preparation for image-based cell counting

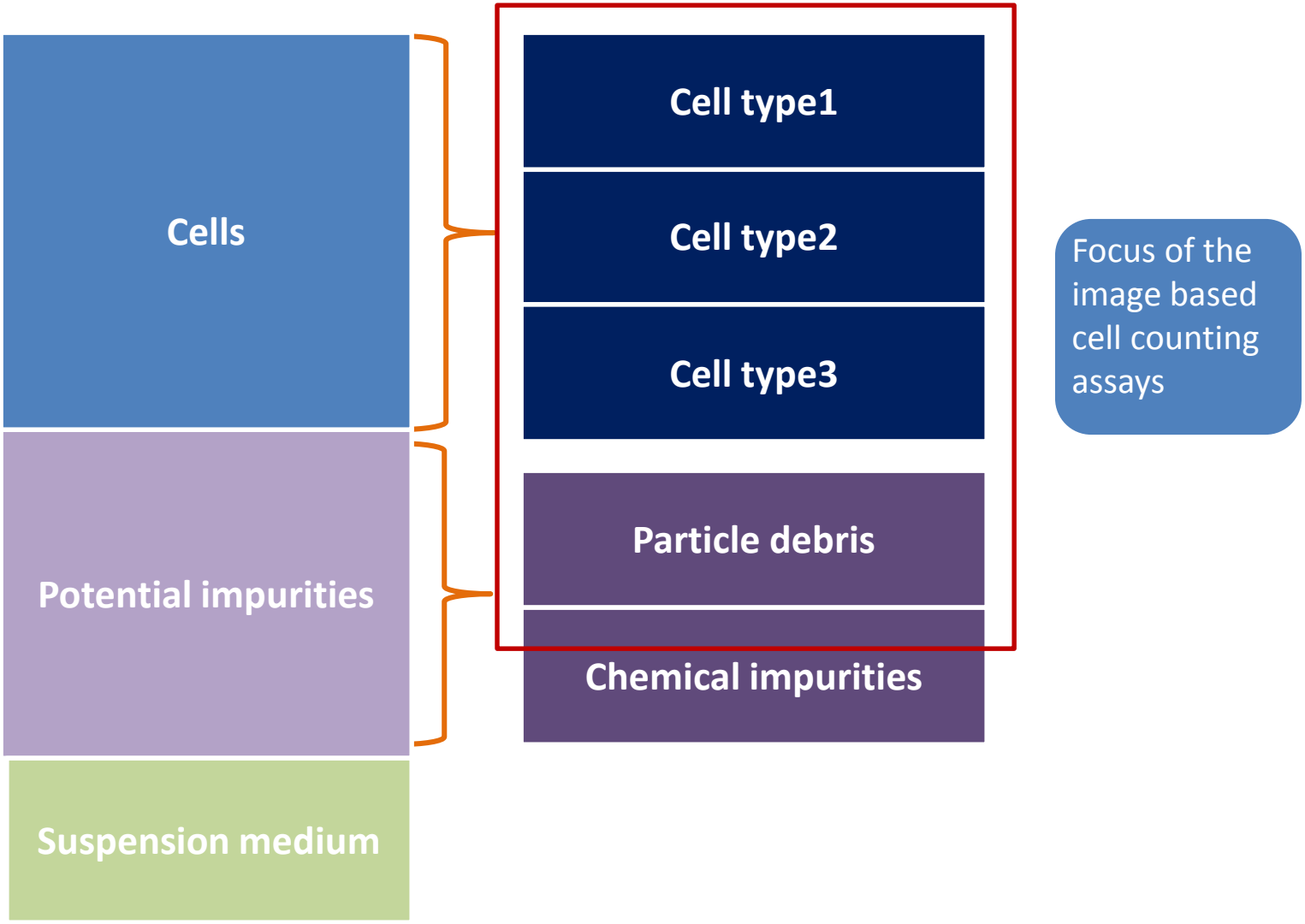
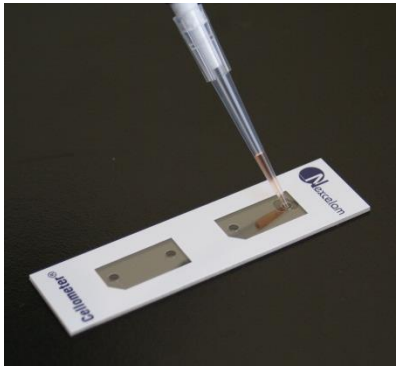
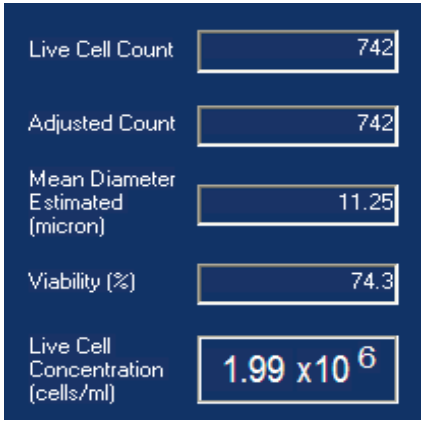


Image-based cell counting principles



1. Pipette 20uL of cells into disposable counting chamber

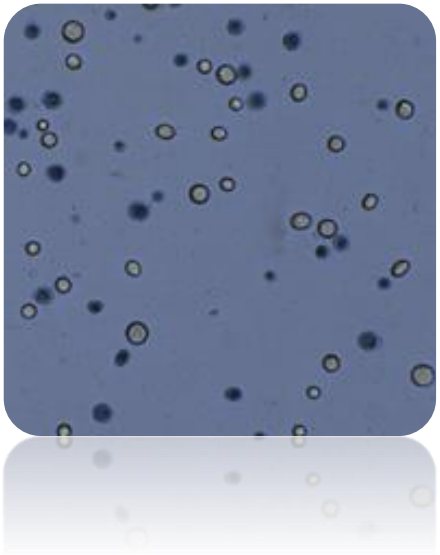
2. Insert chamber in Cellometer and click count



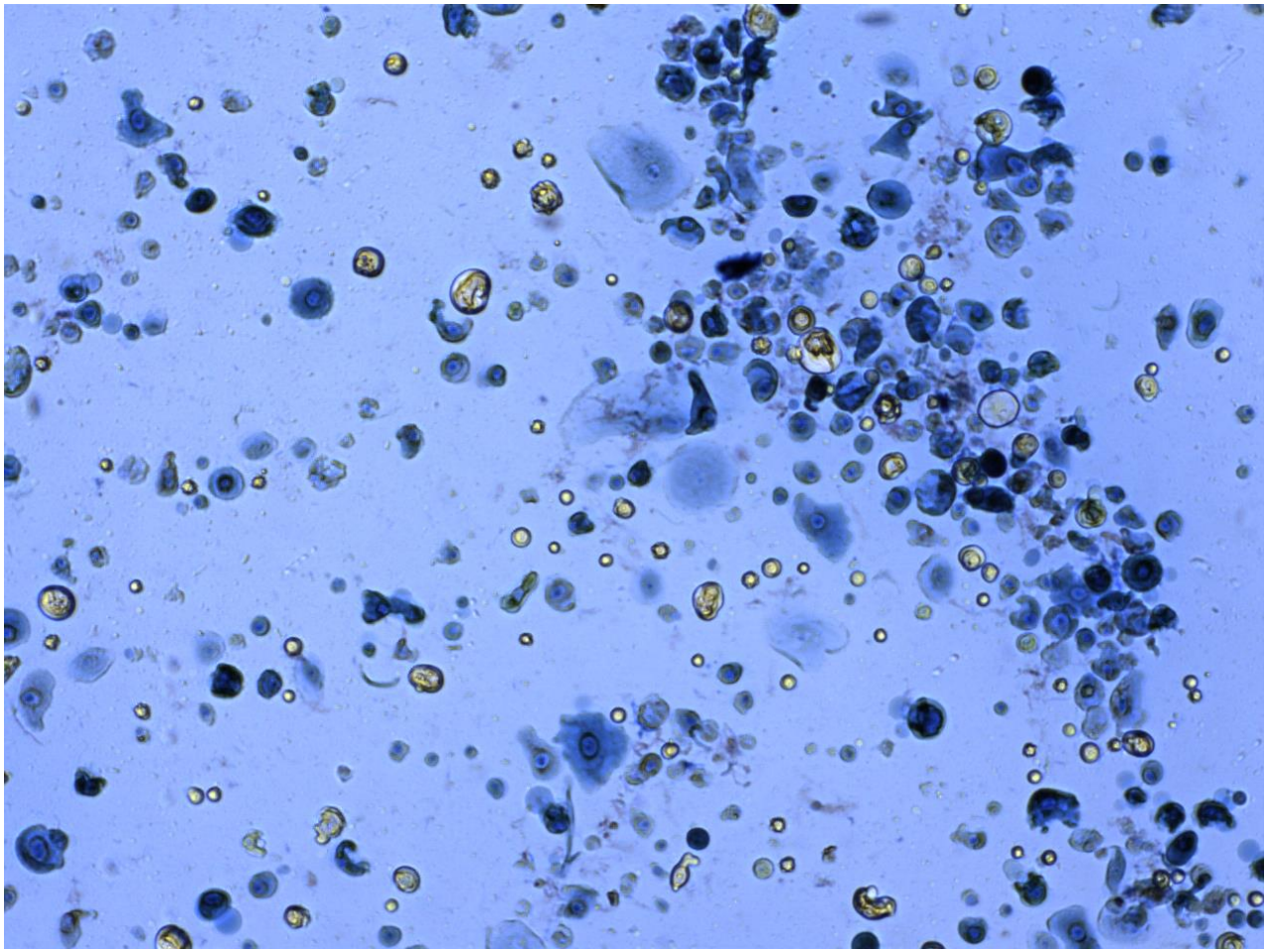
3. Cell count data is generated automatically

Cell counting assay using bright-field image

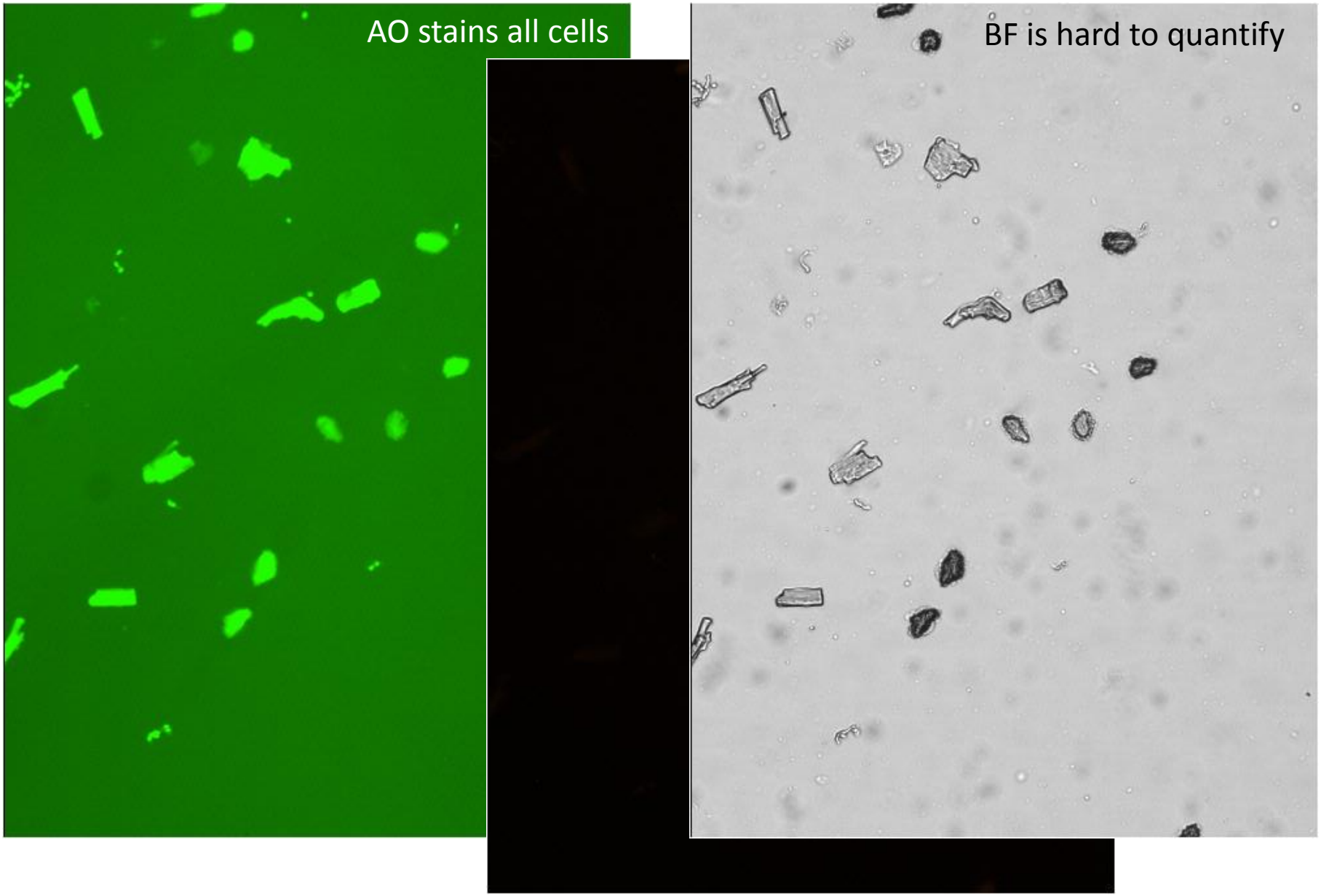
Most of the time you see
this on a product literature



Real life can be messy

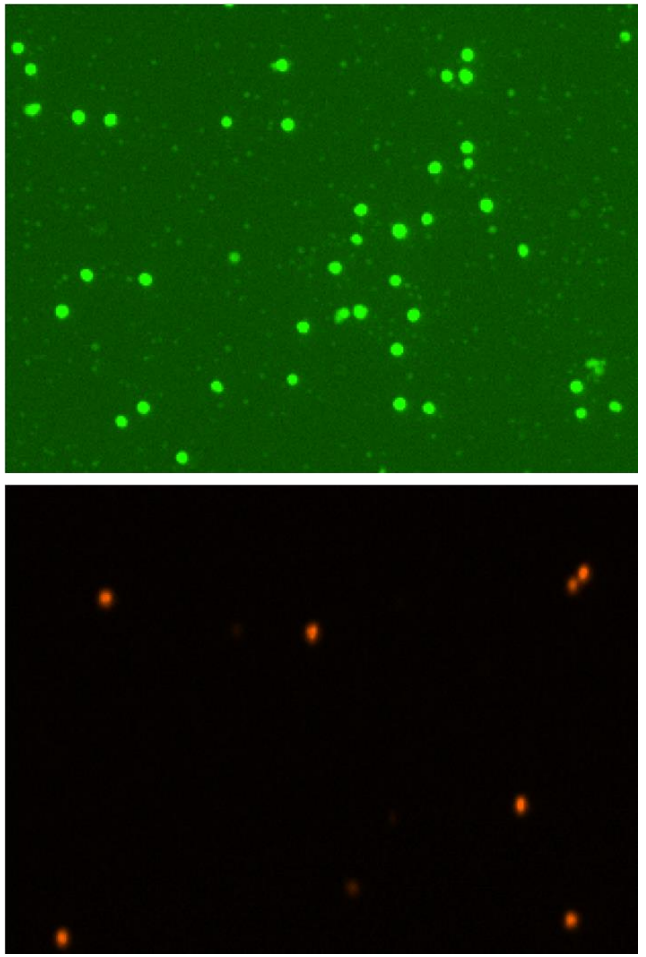


FL staining can be more specific for some cell types - cadiomyocytes



Cell counting assay using dual fluorescence nuclear stains

- AO is permeable to both live and dead cells
- AO binds to DNA and fluoresce bright green
- PI can only enter dead cells
  - Binds to DNA of the dead cells
  - Absorbs the green fluorescence of AO
  - Produces bright orange / red color
- No signal is generated from non- nucleated cells and debris



- Original AO/EB staining protocol developed at Stanford, Prof. Leonard Herzenberg's lab
- Nature Protocols, Vol 8.No. 1, 33, 2013

Identification, isolation and in vitro expansion of human and nonhuman primate T stem cell memory cells

Enrico Lugli, Luca Gattinoni, Alessandra Roberto, Domenica Mavilio, David A Price, Nicholas P Restifo & Mario Roederer

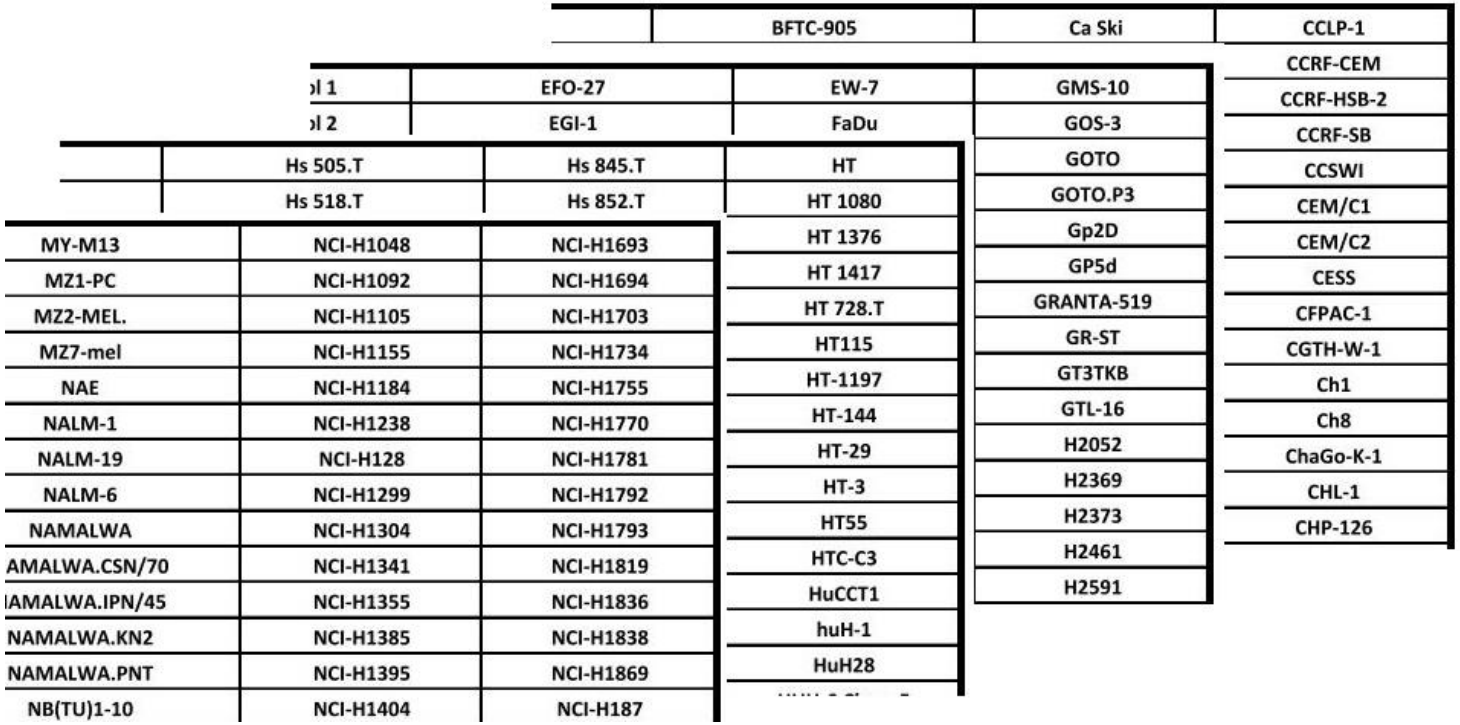
Cell counting fit-for-purpose case I: oncology screening projects using human cell line panels

NCI 60 cancer cell line panel

- NCI-60 cancer cell line panel defined by National Cancer Institute
- Used for screening of promising cancer therapeutics
- Over 3,000 distinct compounds screened each year

Source	Cell Lines
Lung	NCI-H23, NCI-H522, A549-ATCC, EKVX, NCI-H226, NCI-H332M, H460, H0P62, HOP92
Colon	HT29, HCC-2998, HCT116, SW620, COLO205, HCT15, KM12
Breast	MCF7, MCF7ADRr, MDAMB231, HS578T, MDAMB435, MDN, BT549, T47D
Ovarian	OVCAR3, OVCAR4, OVCAR5, OVCAR8, IGROV1, SKOV3
Leukemia	CCRFCEM, K562, MOLT4, HL60, RPMI8266, SR
Renal	UO31, SN12C, A498, CAKI1, RXF393, 7860, ACHN, TK10
Melanoma	LOXIMVI, MALME3M, SKMEL2, SKMEL5, SKMEL28, M14, UACC62, UACC257
Prostate	PC3, DU145
CNS	SNB19, SNB75, U251, SF268, SF295, SM539

Much larger cell line panels



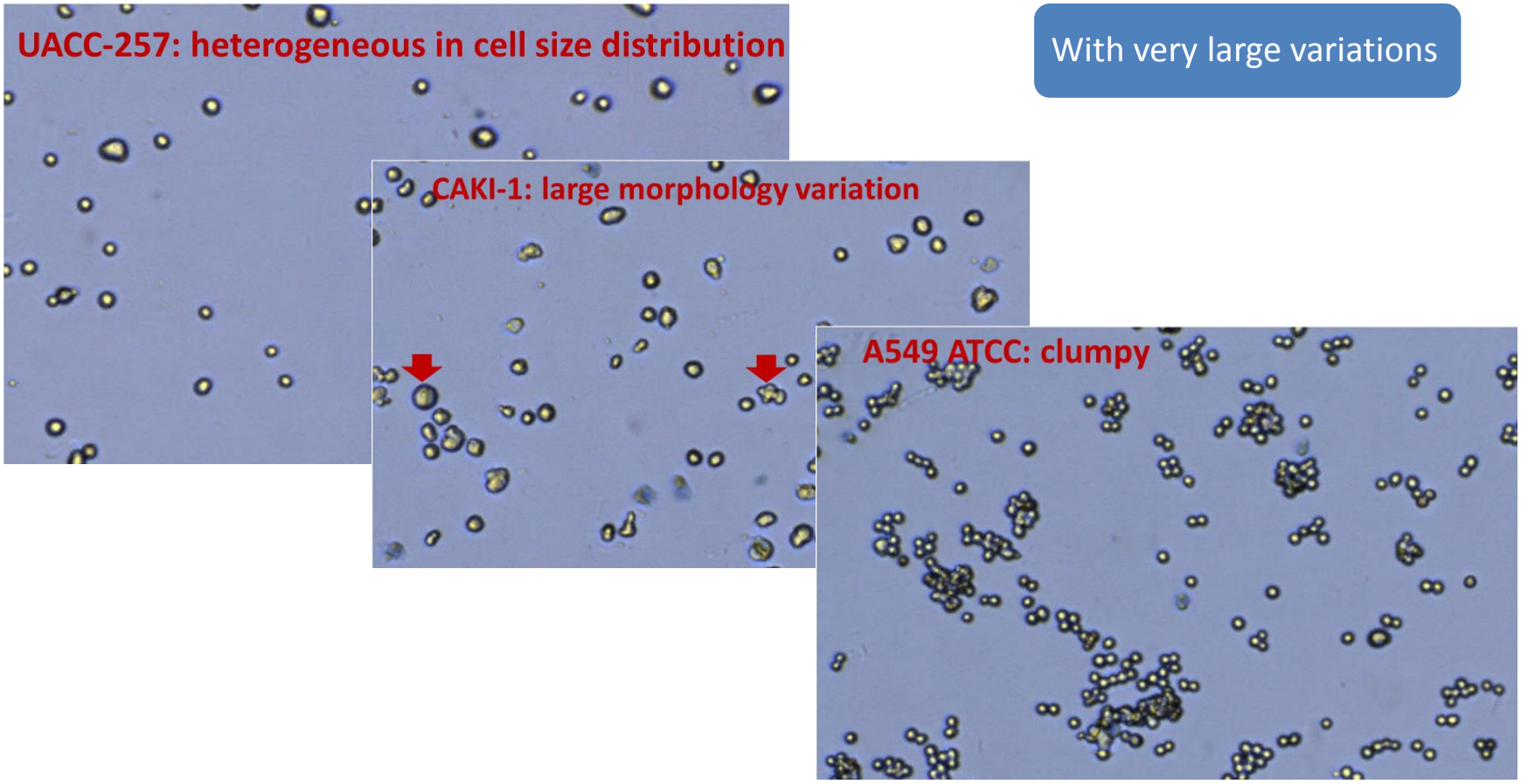
			BFTC-905	Ca Ski	CCLP-1
					CCRF-CEM
		ol 1	EFO-27	EW-7	GMS-10
		ol 2	EGI-1	FaDu	GOS-3
					CCRF-SB
	Hs 505.T	Hs 845.T	HT	GOTO	CCSWI
	Hs 518.T	Hs 852.T	HT 1080	GOTO.P3	CEM/C1
MY-M13	NCI-H1048	NCI-H1693	HT 1376	Gp2D	CEM/C2
MZ1-PC	NCI-H1092	NCI-H1694	HT 1417	GP5d	CESS
MZ2-MEL.	NCI-H1105	NCI-H1703	HT 728.T	GRANTA-519	CFPAC-1
MZ7-mel	NCI-H1155	NCI-H1734	HT115	GR-ST	CGTH-W-1
NAE	NCI-H1184	NCI-H1755	HT-1197	GT3TKB	Ch1
NALM-1	NCI-H1238	NCI-H1770	HT-144	GTL-16	Ch8
NALM-19	NCI-H128	NCI-H1781	HT-29	H2052	ChaGo-K-1
NALM-6	NCI-H1299	NCI-H1792	HT-3	H2369	CHL-1
NAMALWA	NCI-H1304	NCI-H1793	HT55	H2373	CHP-126
AMALWA.CSN/70	NCI-H1341	NCI-H1819	HTC-C3	H2461	
AMALWA.IPN/45	NCI-H1355	NCI-H1836	HuCCT1	H2591	
NAMALWA.KN2	NCI-H1385	NCI-H1838	huH-1		
NAMALWA.PNT	NCI-H1395	NCI-H1869	HuH28		
NB(TU)1-10	NCI-H1404	NCI-H187			

- Novartis /Broad (CCEL) - 1,000 cell types
- MGH/Sanger – more than 1,600 cell types

Purpose of the cell counting

- Count cells for cell line expansion
- Count cells to seed assay plates (1536w, 384w, 96w)

What do cell preparations look like for NCI-60 cell lines?

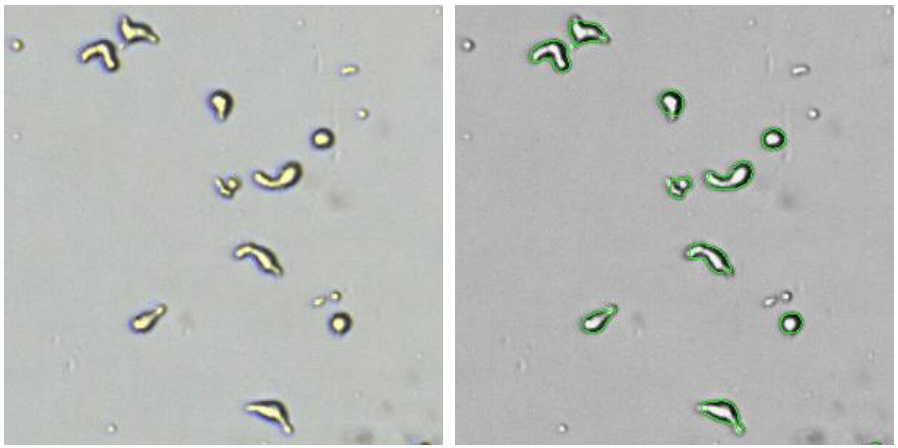


With very large variations

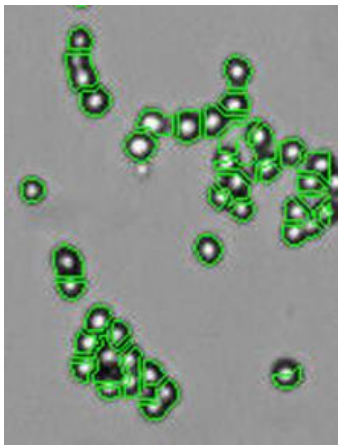
Summary of NCI 60 diverse morphologies

Cell Characteristics	Description (trypsinized)	% Total
Round with narrow size distribution	Cells with round shape, not sticking to each other Not a very large size distribution	43%
Clumpy	Cells stick to each other forming clumps, typically more than 5 cells per clump	57%
Clustering	Cells stick to each other forming clusters, typically less than 5 cells per cluster	
Debris	Dead cells form debris in suspension	
Large Morphology Variation	Cells vary in size and shape	

Cell Membrane Outline Algorithm to address the morphological diversity for counting



Algorithms Identify irregular shapes
(SK28 cell line)



Algorithms identify
cell clusters

Advanced imaging and analysis algorithms are necessary to address the cell morphology diversity in the large tumor cell lines.

Case I summary

- Each cell preparation contains only one cell type
- Cell preparation is generally in heathy conditions with viability higher than 90%
- Bright-field based cell counting assay can be used
- Special image analysis capability is required for devise morphologies

Cell counting fit-for-purpose case II: cell therapy bio-processing

Examples of cell therapy production processes

- CAR-T manufacturing
- Hematopoietic stem cell
- Therapeutic cancer vaccine

Cancer Gene Therapy (2015) 22, 85–94

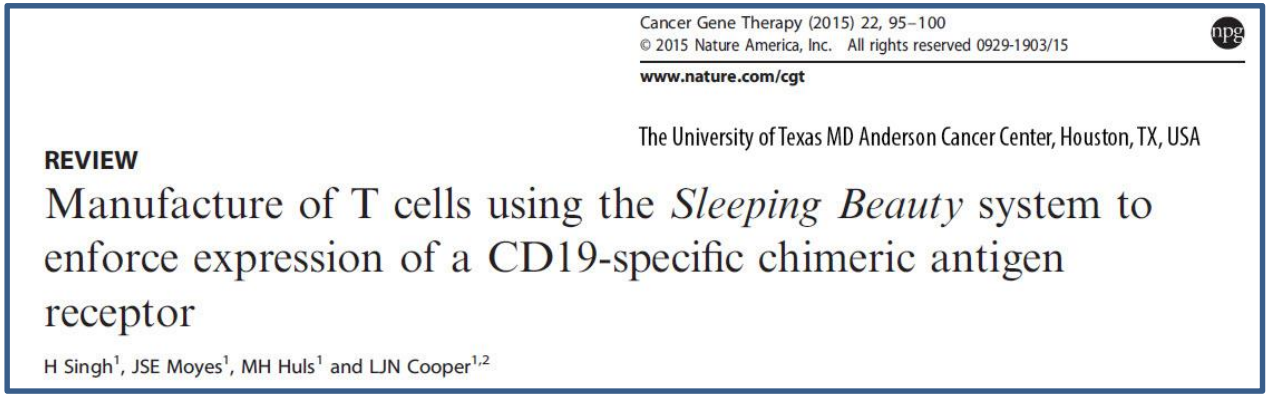
www.nature.com/cgt

REVIEW

Cell Therapy and Cell Engineering Facility, Memmorial Sloan Kettering Cancer Center, New York

Manufacture of tumor- and virus-specific T lymphocytes for adoptive cell therapies

X Wang[1,2] and I Rivière[1,2,3]



Cancer Gene Therapy (2015) 22, 95–100

www.nature.com/cgt

The University of Texas MD Anderson Cancer Center, Houston, TX, USA

REVIEW

Manufacture of T cells using the *Sleeping Beauty* system to enforce expression of a CD19-specific chimeric antigen receptor

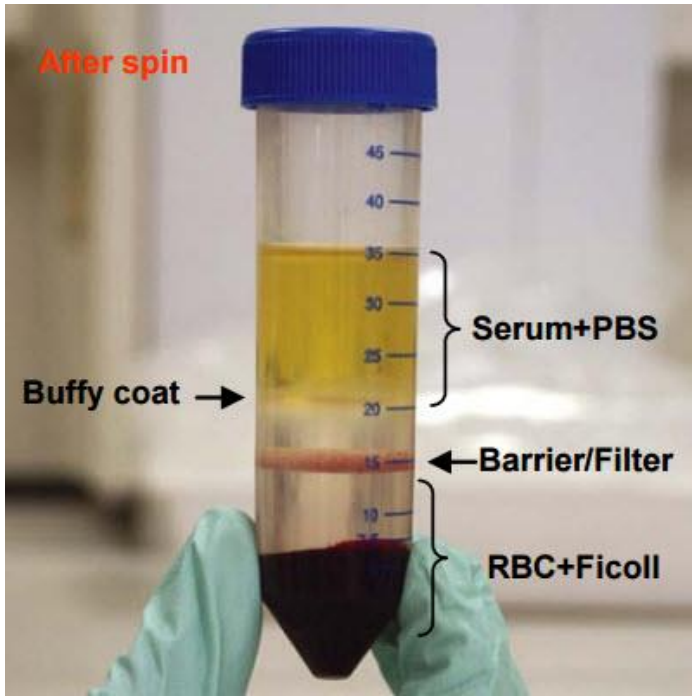
H Singh[1], JSE Moyes[1], MH Huls[1] and LJN Cooper[1,2]

Purpose of the cell counting

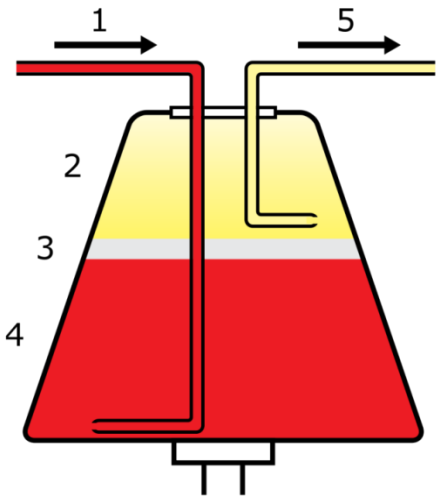
- Count cells from starting material, in-processing material and the final products

Source of the starting material

PBMC by Ficoll separation

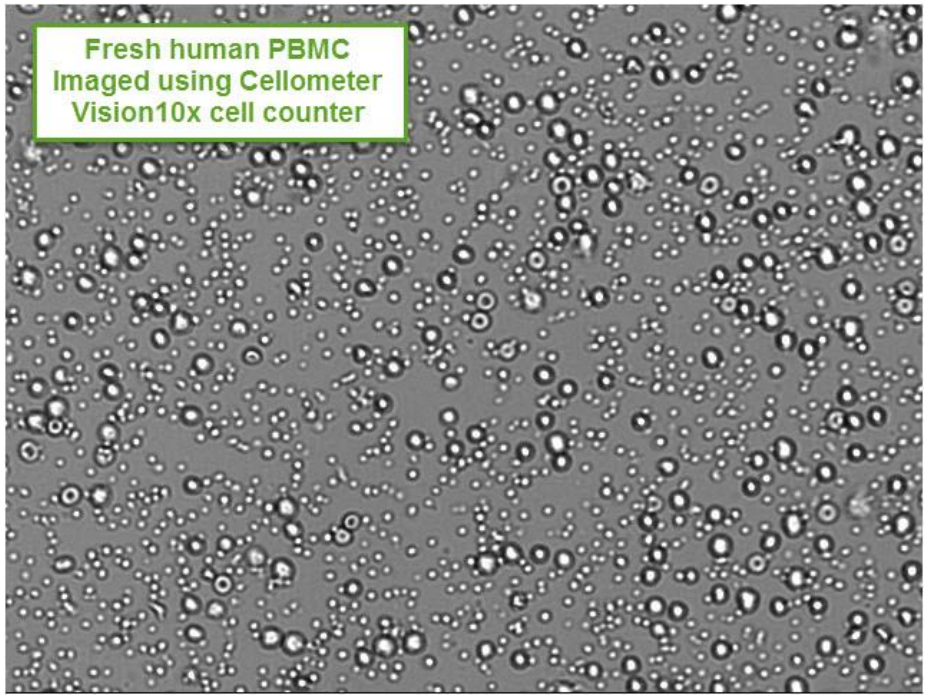


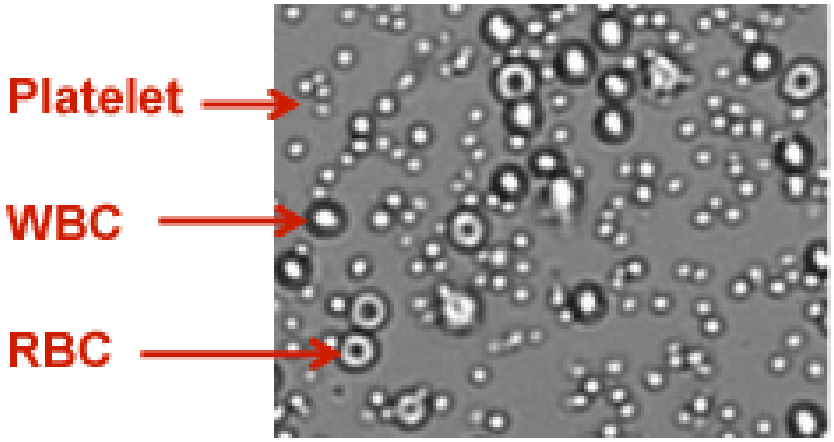
Leukopak from leukophoresis



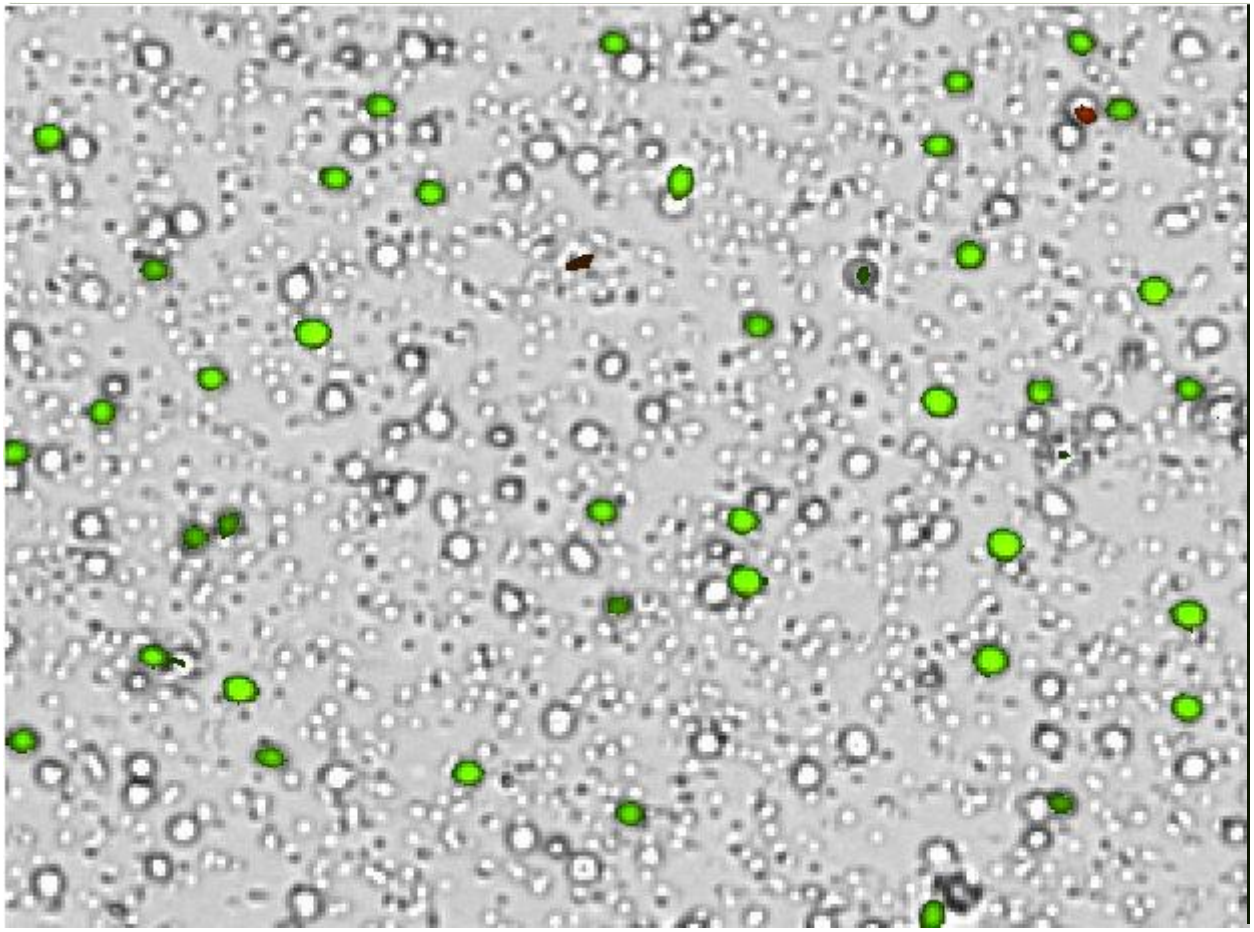
Other source materials: bone marrow, cord blood

What cell types are in the starting material?





Is the bright-field based counting method selective enough for staring materials?



Starting material from Leukopheresis

Total cells: 119

Total FL cells: 39

Over counted cells: 80
They are not nucleated.
Over counting: 3x

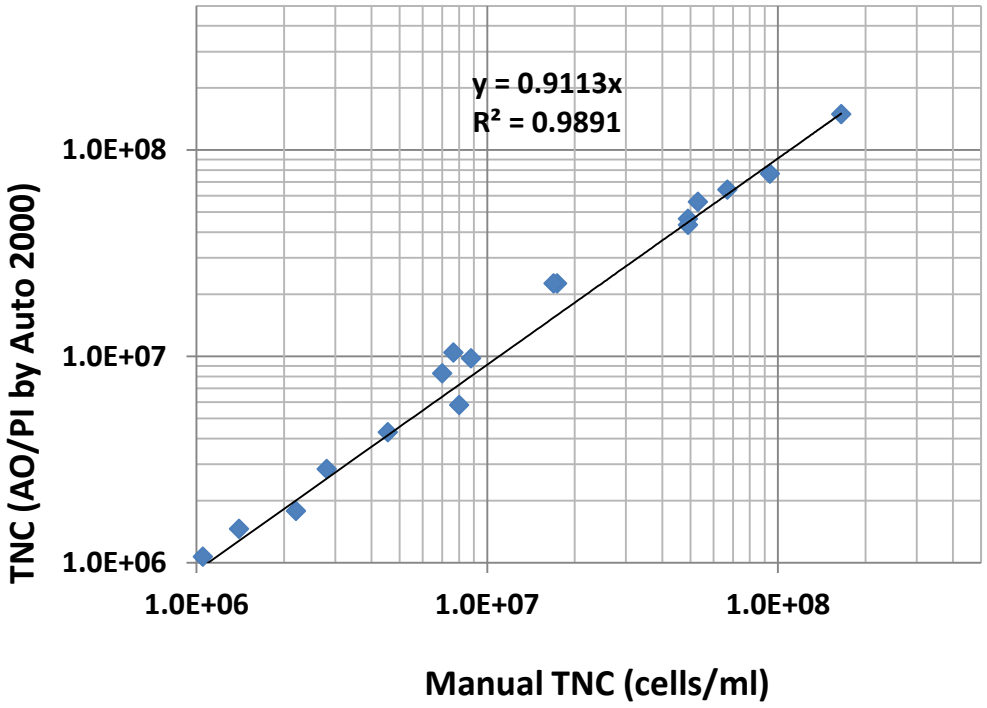
Multiple Leuokopheresis samples shown varied amount of RBC

- Manual counting using Hemacytometer and trypan blue
- Automatic cell counting using Cellometer Vision and AO/PI stains
- Experiment using Leukopheresis samples

Sample ID	**A**	**B**	**C**	**D**	**E**	**F**
Ratio (Manual counting to Cellometer AO/PI)	**7**	**5**	**12**	**3**	**7**	**5**

TNC using manual and automatic cell counting with good correlation

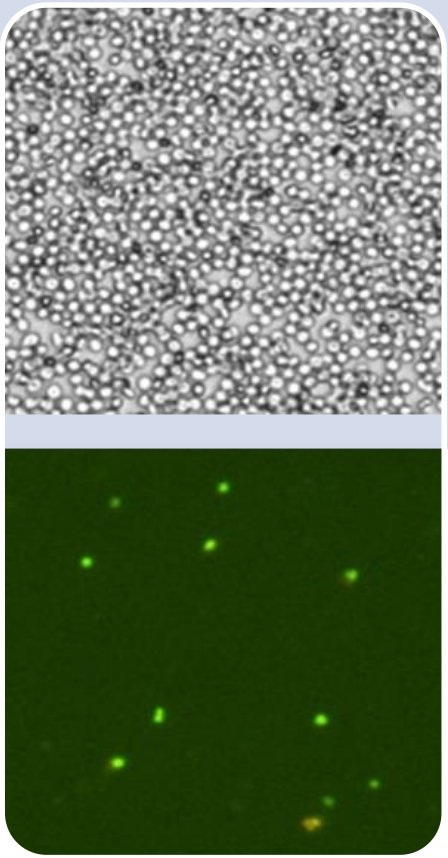
- Manual counting using Hemacytometer post lysing red blood cells
- Automatic cell counting directly stain starting material with AO/PI prior to cell counting
- Starting materials were bone marrow, leukopak, cord blood

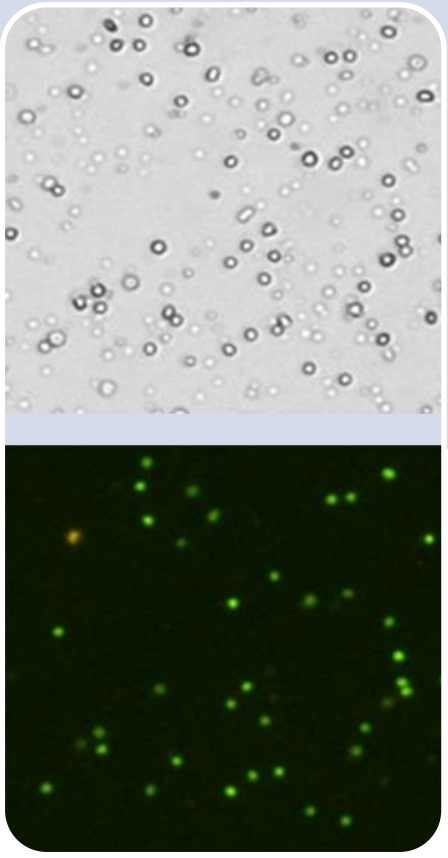


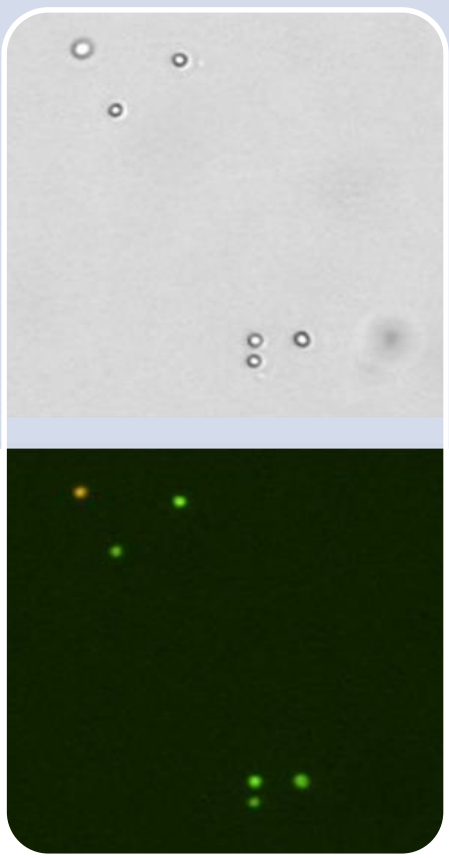
Select a cell counting system with multiple cell counting assays

Bright-field image

Fluorescent image







Source materials:
PB, BM, CB

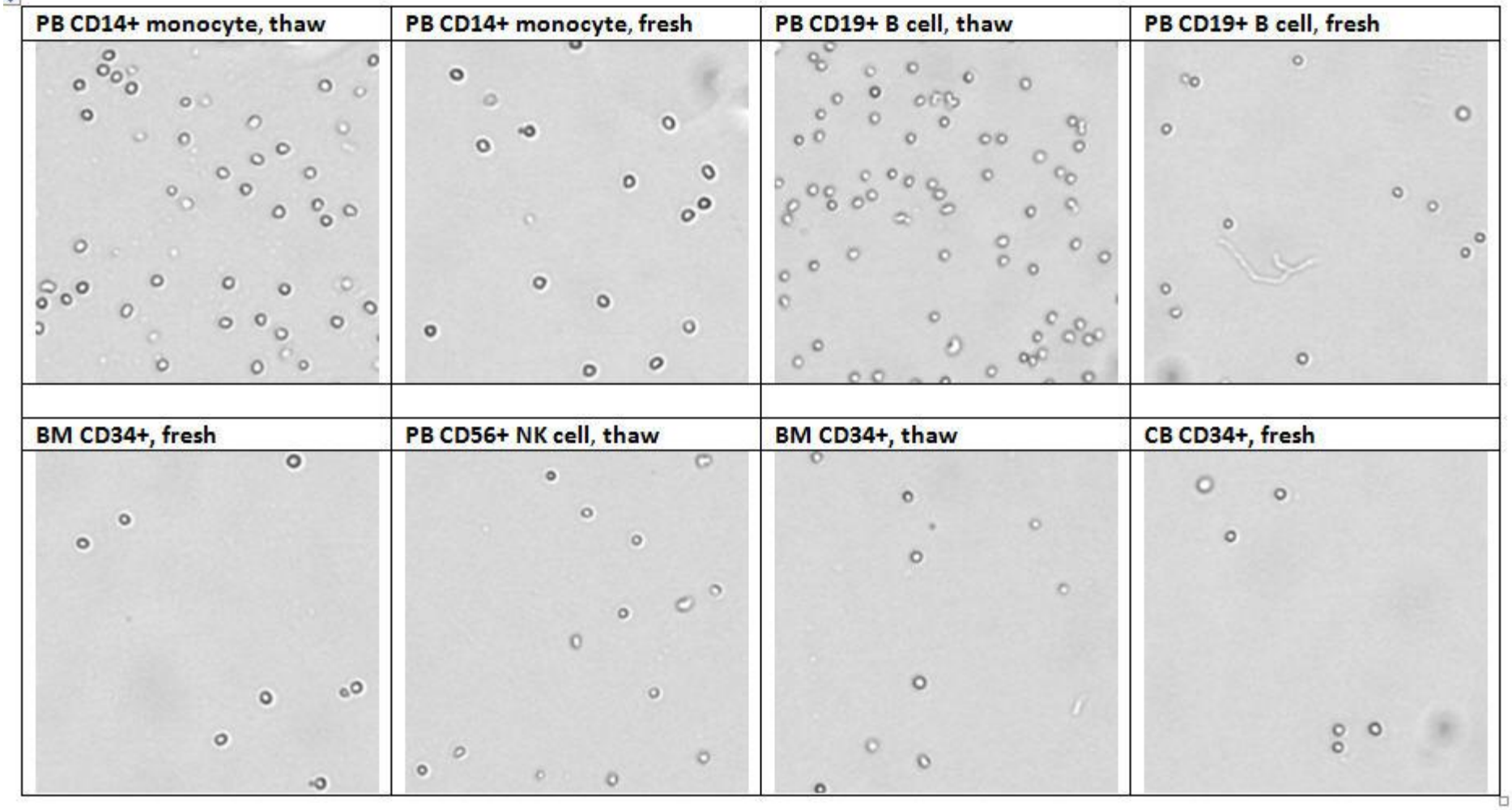
In processing:
MNC

Finished product:
CD34+ cells

AO/PI

Trypan blue

More finished products with cell preparation containing only one cell type, no particle debris



Bright-field cell counting assay using trypan blue works well with finished products.

Case II summary

- Cell preparation changes though cell process
- Without lysing, fluorescence staining method is more selective
- Bright-field based cell counting assay can be used for finished product when cell preparation is
- Cell counting system provides multiple assays are essential to satisfy varied cell preparation conditions

Cell counting fit-for-purpose case III: primary cells from mouse models

Example cell types and assays from mouse models

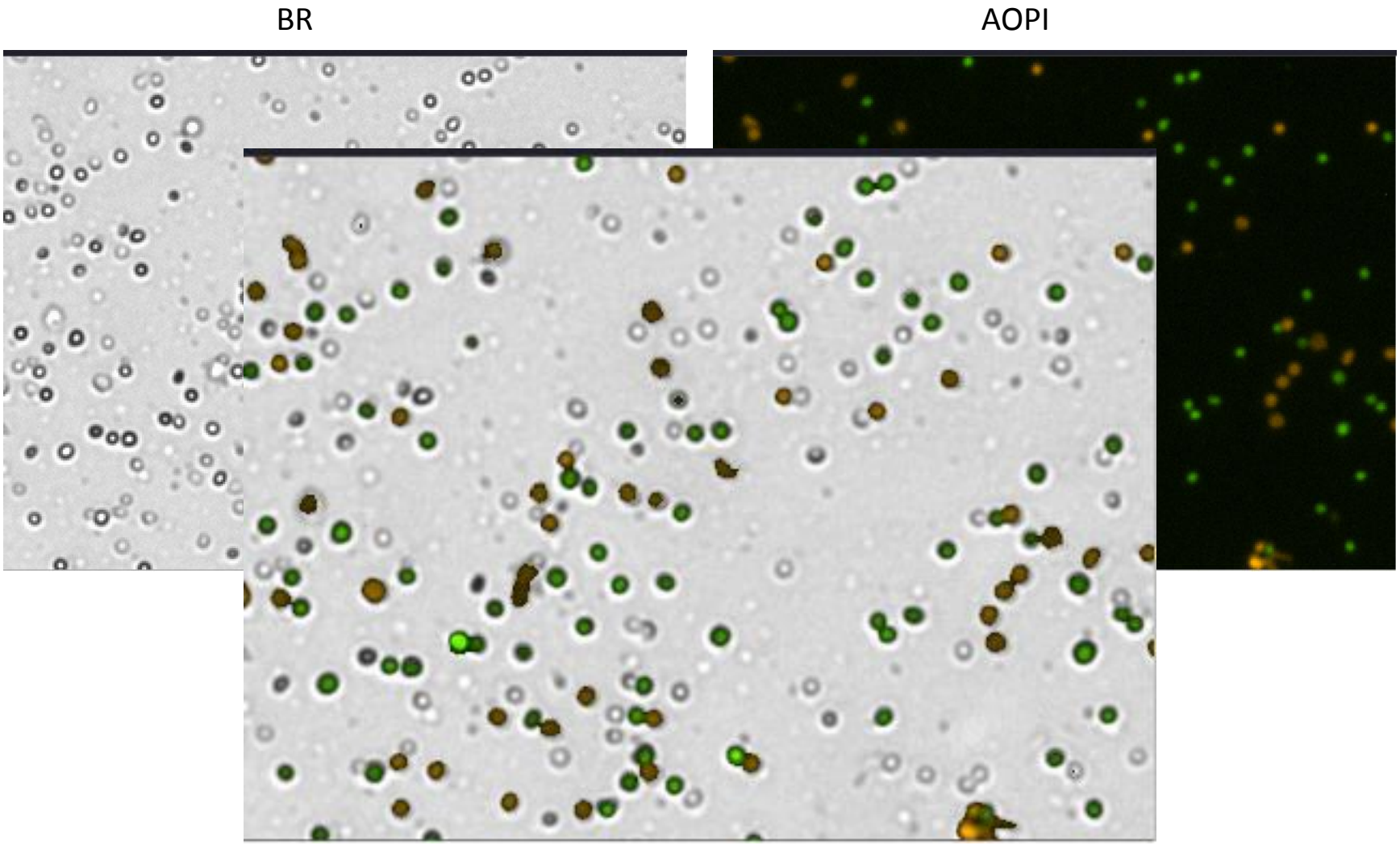
- Tissues
  - Blood, Spleen, BAL, Lung
  - Axillary LN, Mesenteric LN, Inguinal LN, Obturator LN, Iliac (Internal) LN, Cervical LN, Mesenteric LN , Bronchial LN
  - Primary bronchi, Tonsils, Nares Mucosa, Trachea, Rectum, Jejunum, Cervix, Vagina, Uterus, Ileac

- Cells are used for:
  - FACs Surface and Intracellular Staining
  - Elispots
  - qPCR

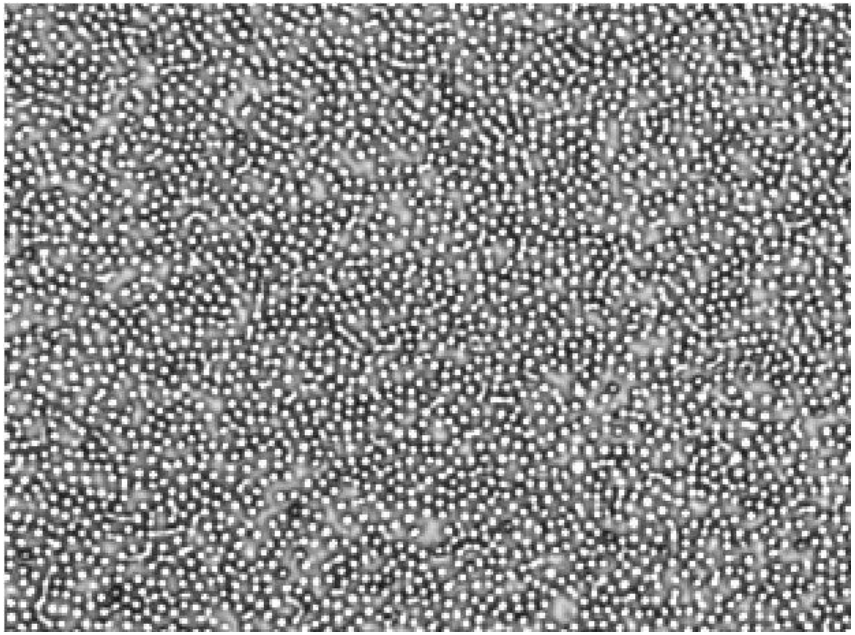
Cell preparation evaluation

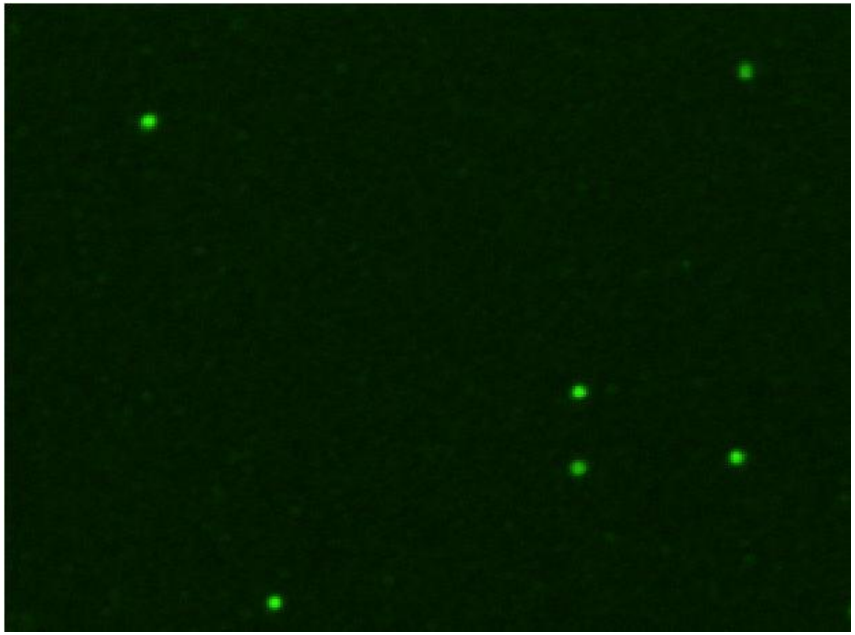
- The operation contains multiple cell preparations
- All cell preparation contains multiple cells types
- Most cell preparation contains particular debris from tissue digestion process
- The amount of cells vary from one animal to another animal

What is in a spleenocyte cell preparation?

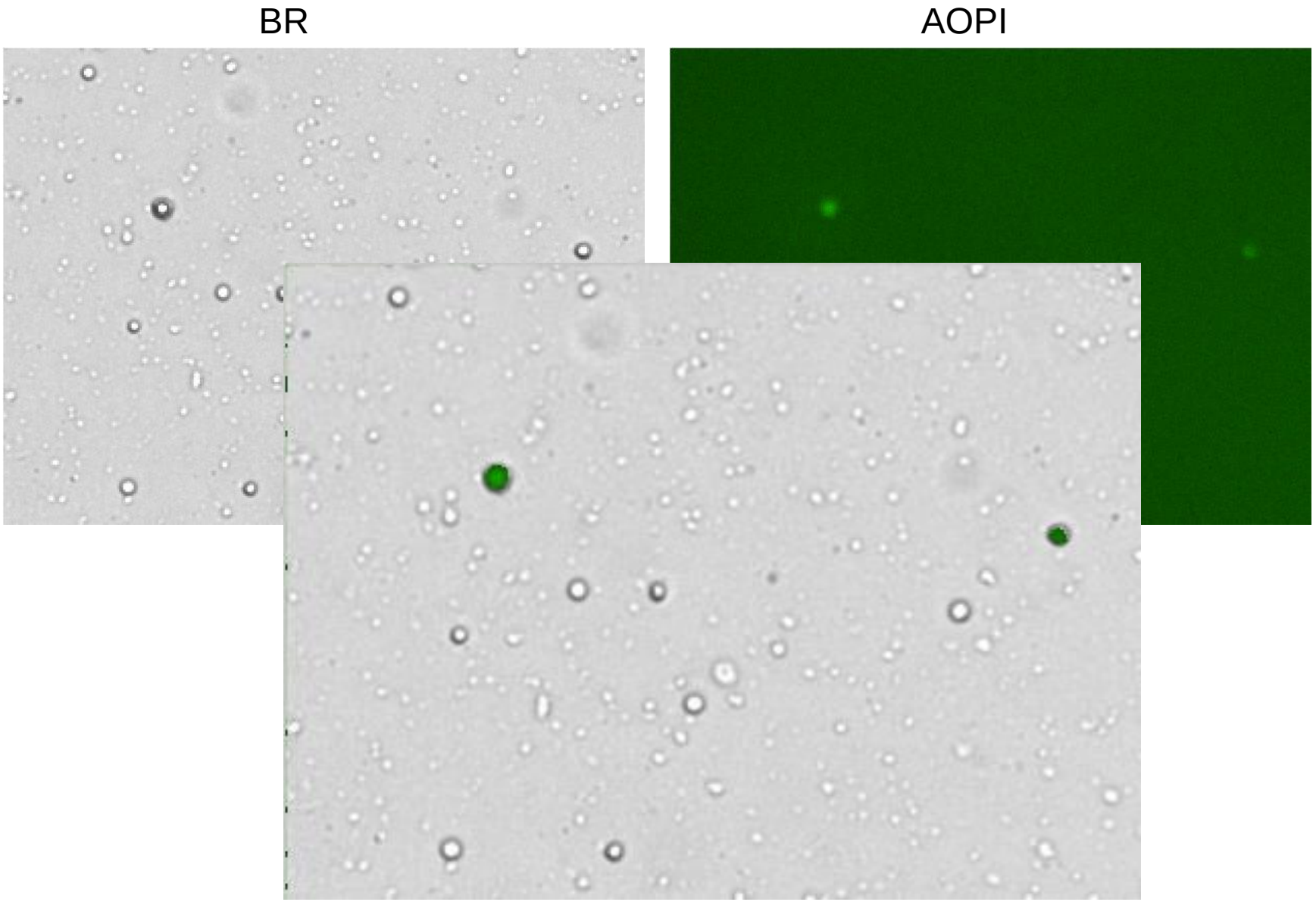


What is in mouse tail blood?



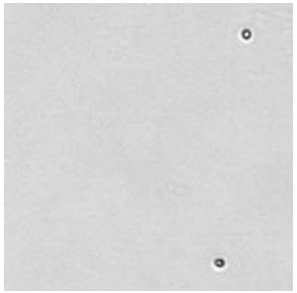
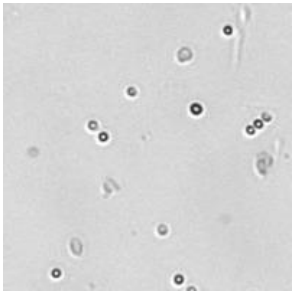
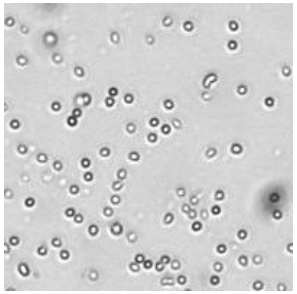
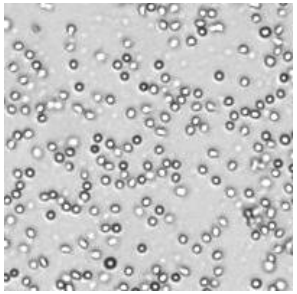
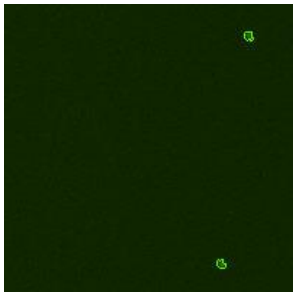
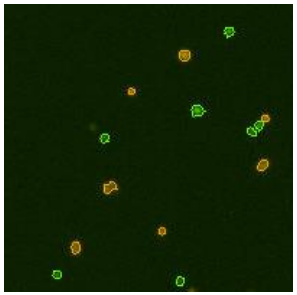
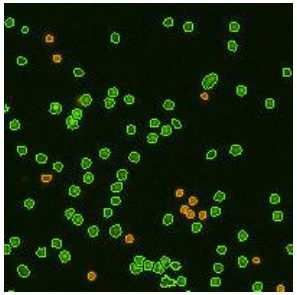
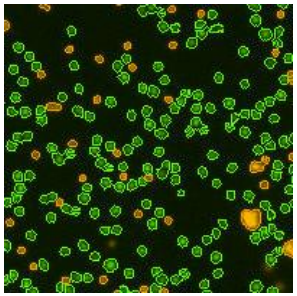


What is in BAL?



Requirements from mouse samples: large dynamic range in both cell count and viability

Results from analysis of 30 distinct lymphocyte samples

	Sample # 7	Sample # 6	Sample # 2	Sample # 5
Total cell count	49	1419	10635	24245
Live cell concentration	1.07E+05	2.84E+06	3.11E+07	6.75E+07
Viability	63.2%	58.0%	84.4%	80.3%
Bright field cell image				
Counted live /dead cell image				

Case III summary

- No tolerance in lysing and other cell processing steps prior to counting – large number of samples per run
- Large variation in the amount and type of particle debris due to tissue source and dissociation processes
- Cell concentration and viability vary in several order of magnitude
- Bright-field based cell counting assay cannot be used
- Dual FL stain provided good cell counting assay

Q&A

Three key fit-for-purpose considerations for cell counting using imaging cytometry

1. Understand cell preparation
2. Evaluate cell counting assays for selectivity
3. Determine tolerance in sample processing

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