Practical Analysis of Stem Cells by Flow Cytometry

Established Methods and Emerging Technologies

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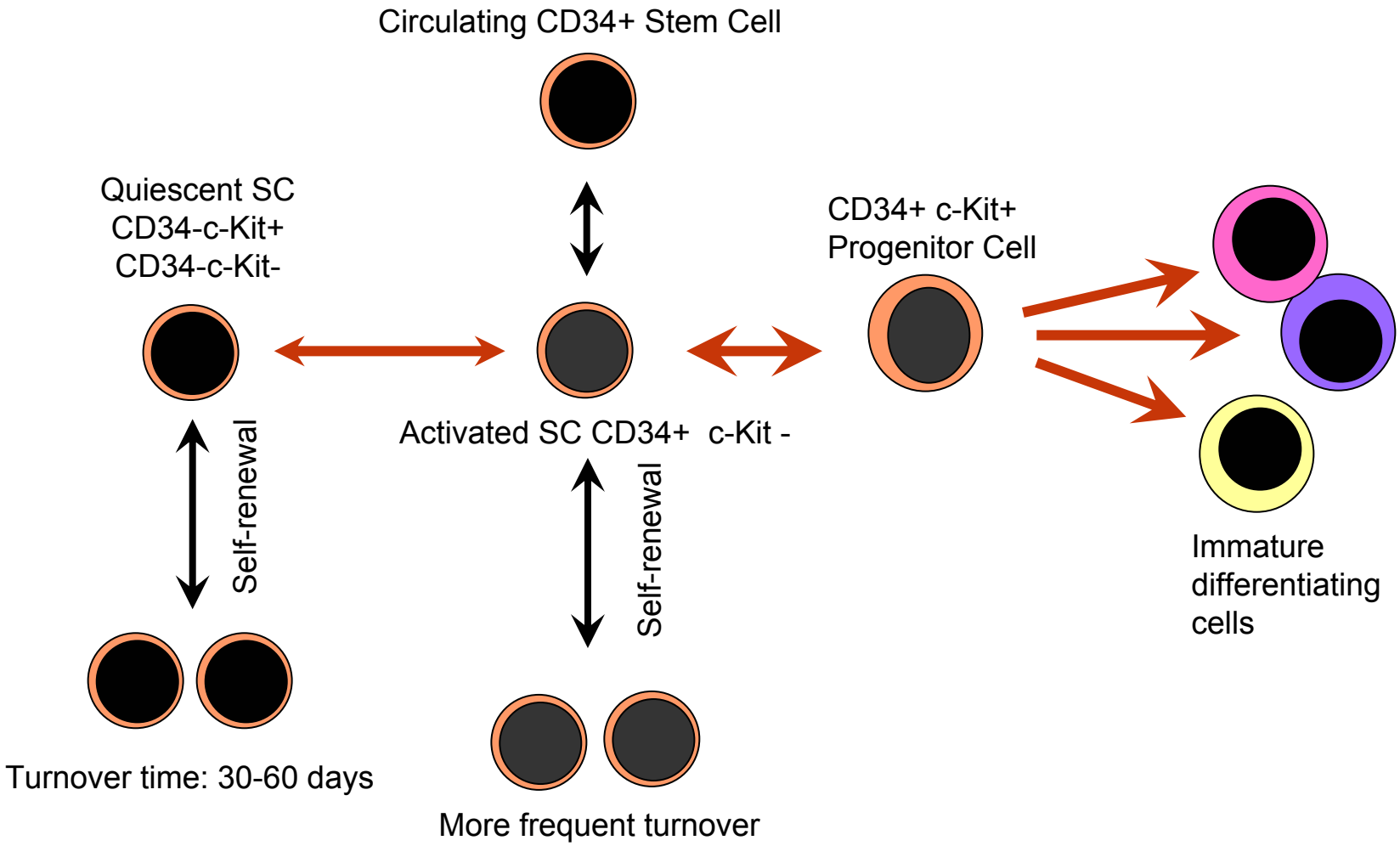
Flow Cytometry in Stem Cell Biology

Flow cytometry and cell sorting are absolutely indispensable techniques for both the identification and isolation of embryonic and adult stem cells in both bone marrow and other tissues.

- Fluorescent immunophenotyping for stem cell markers
- Aldehyde dehydrogenase activity detection using fluorogenic substrates
- G0/G1 discrimination using Hoechst 33342/pyronin Y
- Hoechst side population analysis

Stem cell subpopulations and their identification

Hematopoetic stem cells (HSCs)



From DS Donnely DS and Krause DS, Leukemia and Lymphoma, 40, 221-234 (2001)

Isolation of Stem Cells by Flow Cytometry

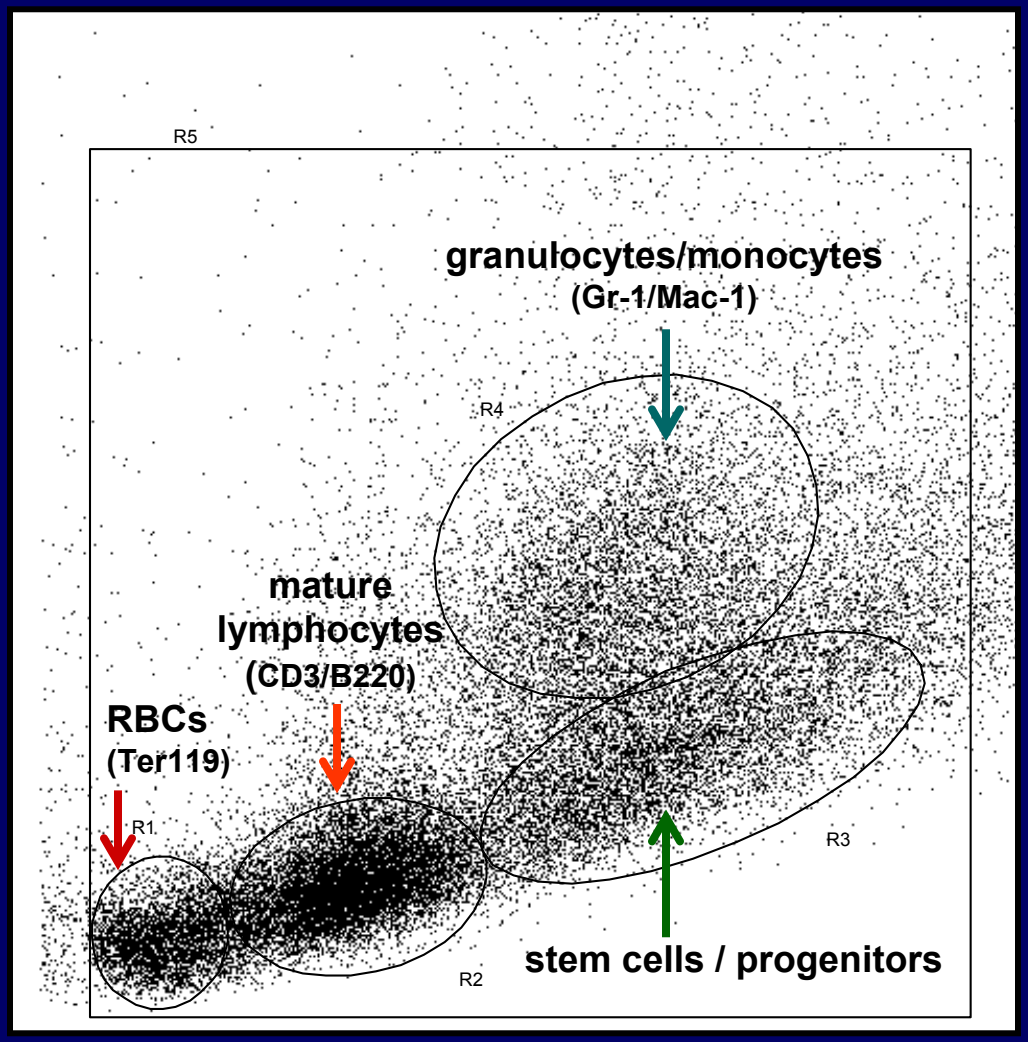
As with most things in biology, there is no single characteristic that adequately identifies stem cells by itself.

We need to look at multiple phenotypic characteristics including cell surface markers and biochemical / physiological characteristics.

And, if possible, we need to look at these multiple characteristics in a single multiparametric assay.

And of course, flow cytometry is an ideal way to do this!

Bone Marrow Cell Differentiation by Scatter Measurement



Phenotypic markers for mammalian hematopoetic stem cells

- **Human stem cells**
  - Lineage depletion
  - CD34
  - HLA-DR
  - AC133 (CD133)
  - CD7
- **Rodent stem cells**
  - Lineage depletion
  - CD34
  - Sca-1
  - c-kit (CD117)
  - MAC-1 (+/-)

Phenotypic markers for mammalian stem cells

CD34

- A single chain transmembrane glycoprotein expressed on HSCs, vascular endothelium, embryonic fibroblasts and some cells in fetal and adult nervous tissue
- Promotes adhesive interactions between stem cells and stromal elements in the bone marrow
- Probably regulates association between stems cells and the “niche” microenvironment via indirect regulation of other adhesion factors
- The dominant marker for clinical identification and separation of stem cells

Phenotypic markers for mammalian stem cells

Sca-1

- Stem cell antigen, a GPI-linked surface protein expressed on 30% of mouse bone marrow including pluripotent HSC and committed lymphoid and myeloid progenitors, also was found in osteoblasts kidney apithelia(Ly-6A/E).
- HSCs from *Sca-1 KO* mice have impaired repopulation potential.
- Lower engraftment of secondary transplants in Sca-1 deficient animals also suggests a defect in HSC self-renewal.
- In addition to HSC deficiencies in Sca-1-deficient mice, specific cell lineages and progenitor subpopulations are also affected. *(Ito CY, Li CY, Bernstein A, Dick JE, Stanford WL. Blood 101, 517-23, 2002).*

Phenotypic markers for mammalian stem cells

c-kit (steel factor)

- Transmembrane glycoprotein, PTK-R in HSC, was found in melanocytes and primordial germ cells
- Mice lacking the receptor tyrosine kinase c-Kit (c-Kit$^{W/W}$) have hematopoietic defects causing perinatal death
- A viable c-Kit$^{W/W}$ mouse shows an age-dependent, progressive decline of pro-T and pro-B cells accompanied by loss of common lymphoid progenitors in the bone marrow in adult mice lacking c-kit.
- Adult c-KitW/W hematopoietic stem cells can engraft in host bone marrow but fail to radioprotect, form spleen colonies, or establish sustained lymphopoiesis *(Waskow C, Paul S, Haller C, Gassmann M, Rodewald H., Immunity 3, 277-88, 2003)*

Benchtop analyzers

- BD FACScan – one laser, three colors
- BD FACScalibur – two lasers, four colors
- Beckman Coulter EPICS XL – one laser four colors

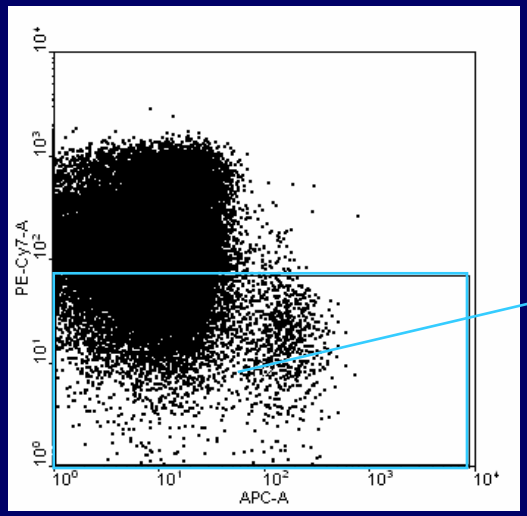
Sca-1 and c-kit expression in mouse bone marrow

Three color analysis

Lineage depletion with biotinylated antibodies against B220, CD3, Ly6C+G, GR-1, Ter119, CD5 and NK1.1

FITC “dump” channel

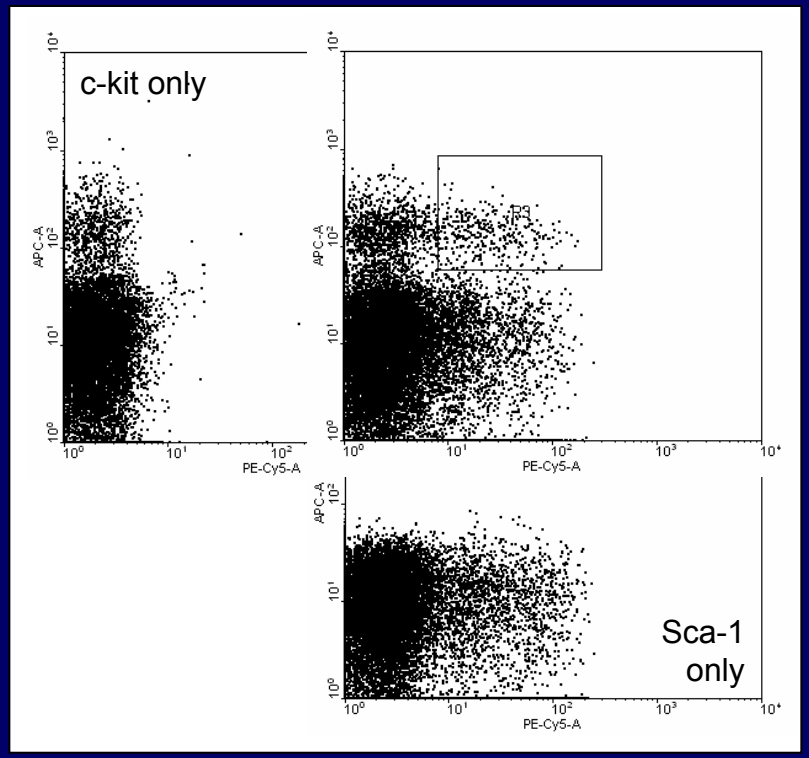
APC-anti-c-kit
PE-Cy5-anti-Sca-1
FITC-Lineage



FITC-lineage

APC-c-kit

APC-C-kit



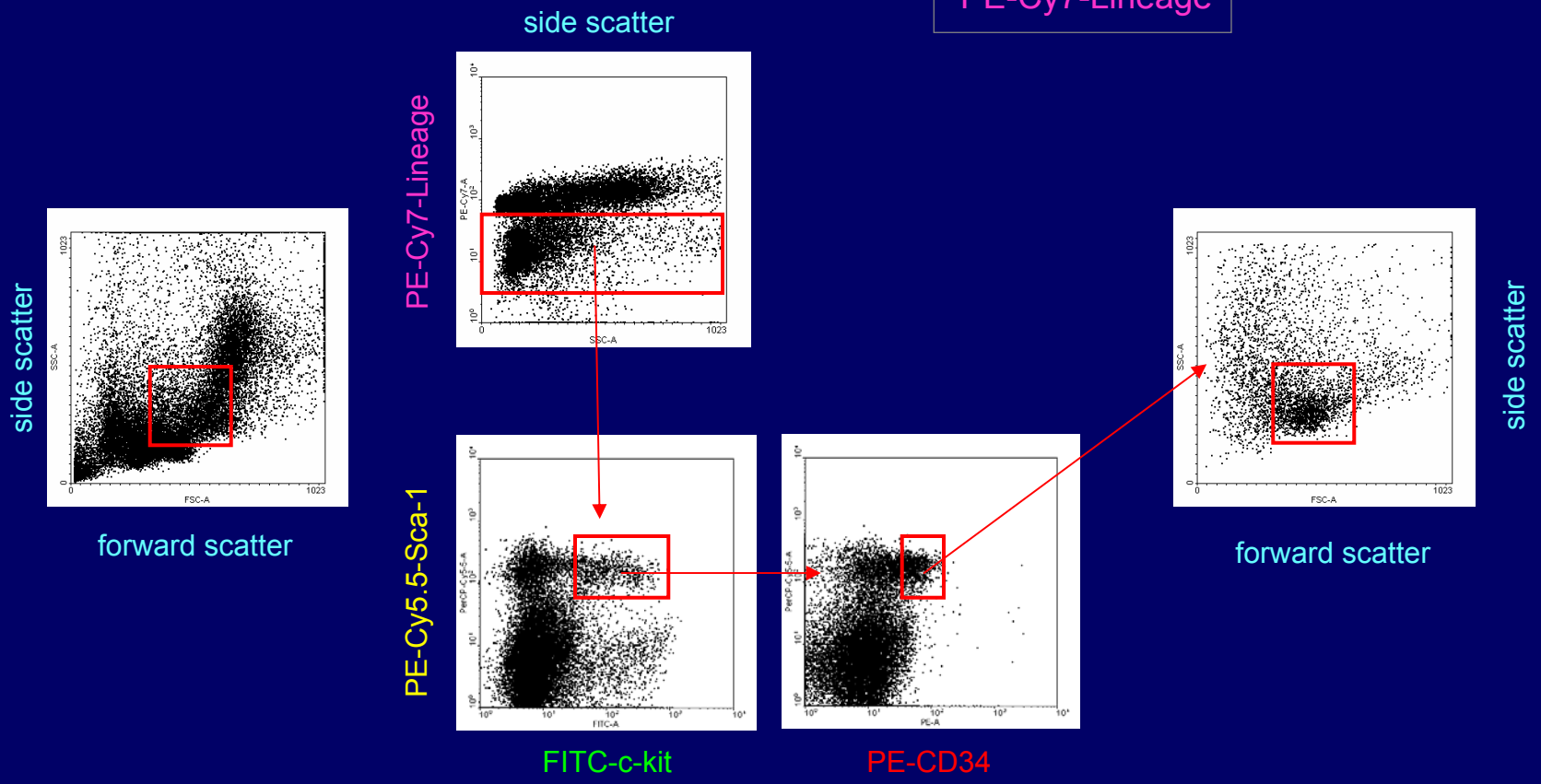
PE-Cy5-Sca-1

Sca-1, c-kit and CD34 expression in mouse bone marrow

Four color analysis

PE-Cy7 labeling for lineage panel
(CD3, B220, Ly6C + G, CD11b, Ter119, NK1.1, CD5)

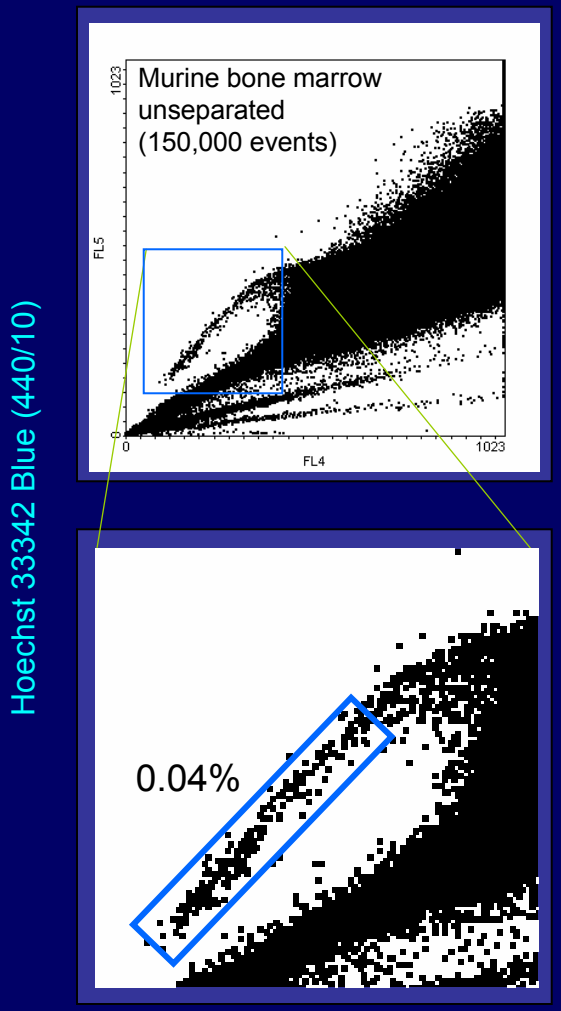
FITC-c-kit
PE-CD34
PE-Cy5-Sca-1
PE-Cy7-Lineage



Discrimination of the Hoechst SP on the flow cytometer

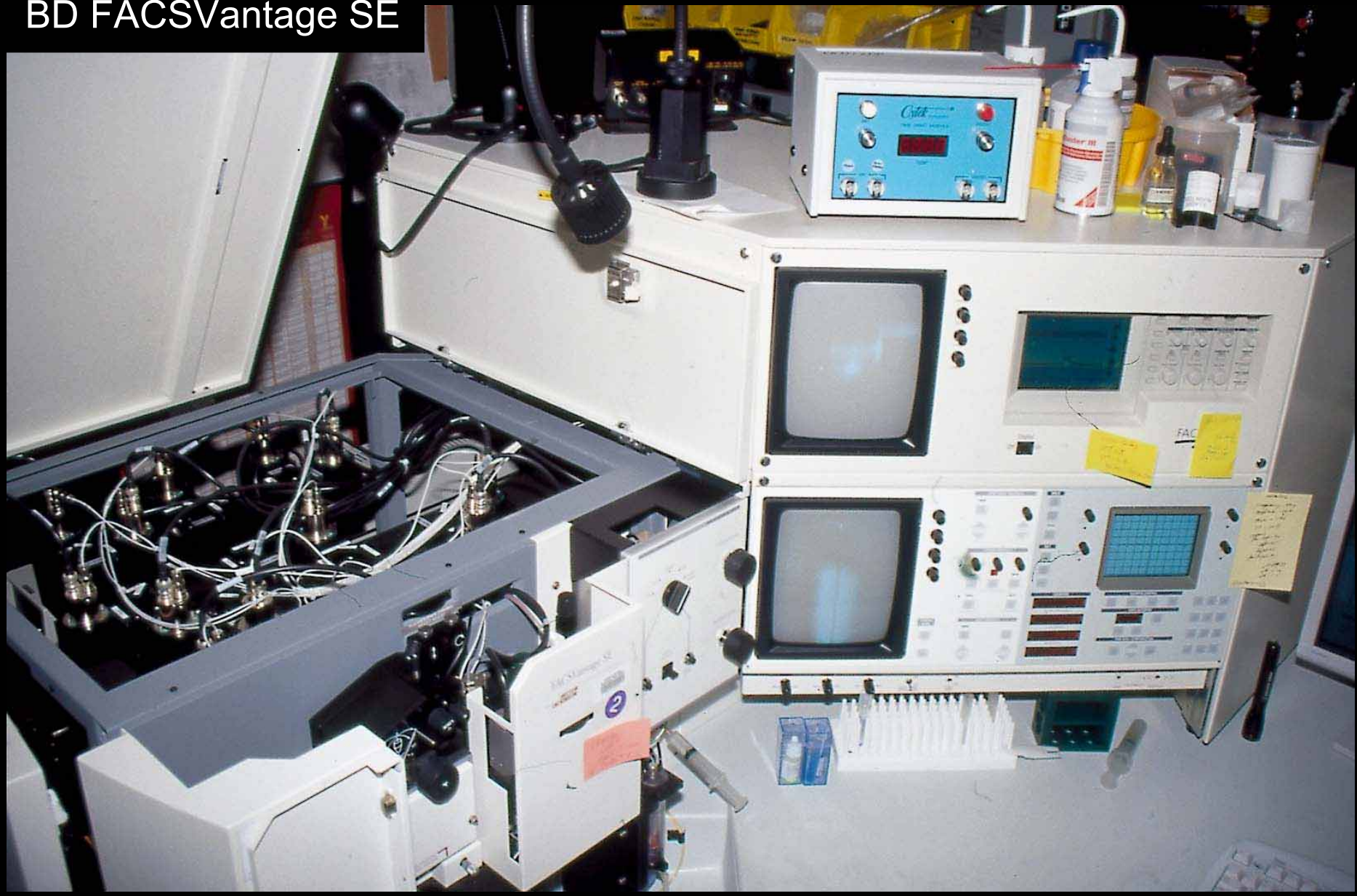
- When loaded with the fluorescent DNA dye **Hoechst 33342**, murine bone marrow stem cells preferentially pump out the dye via the ABCG2 membrane transporter.

- These “side population” (SP) cells can be distinguished on the basis of their reduced Hoechst dye fluorescence (Goodell et al., JEM 183, 1797 (1996)).
- Both DNA-bound (Hoechst blue fluorescence) and unbound (Hoechst red fluorescence) used to distinguish side population cells.
- SP cells are enriched for Sca-1 expression and show no lineage marker expression. SP cells can reconstitute irradiated mice with both lymphoid and myeloid lineages.

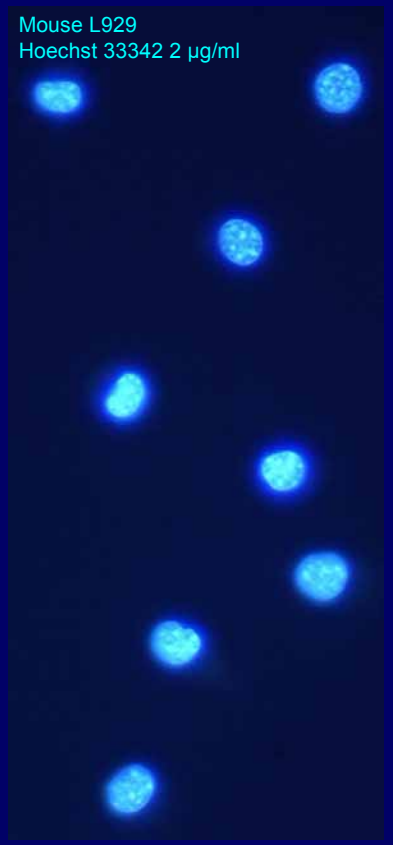


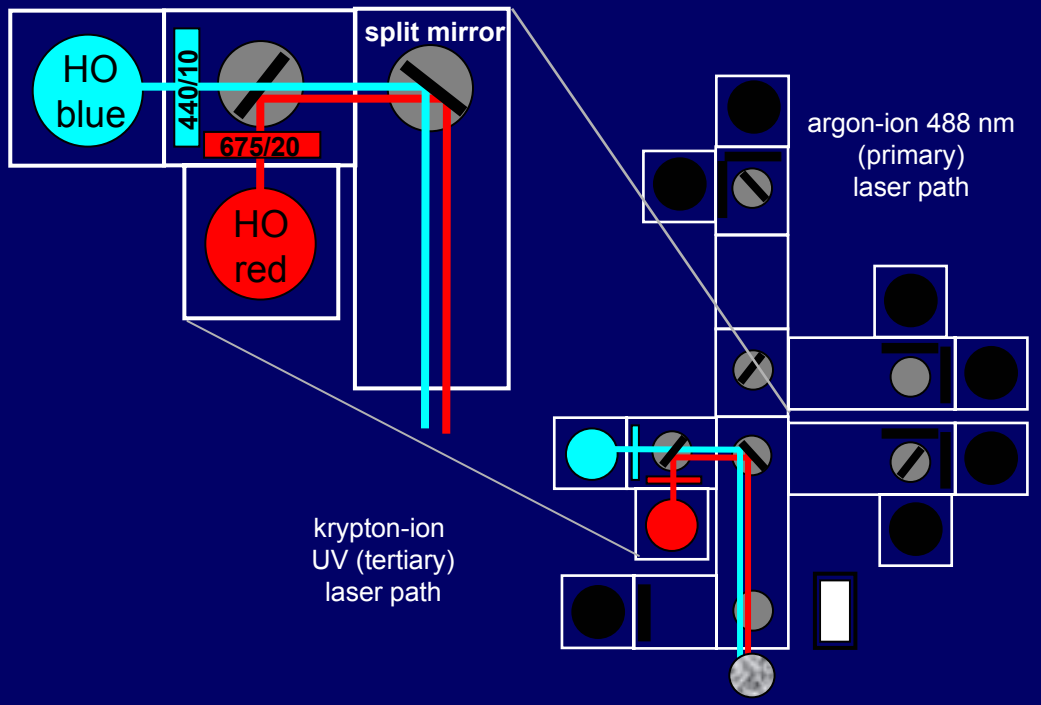
Samples from Drs. Atsushi Terasumi and John Vogel, DB, NCI, NIH

BD FACSVantage SE

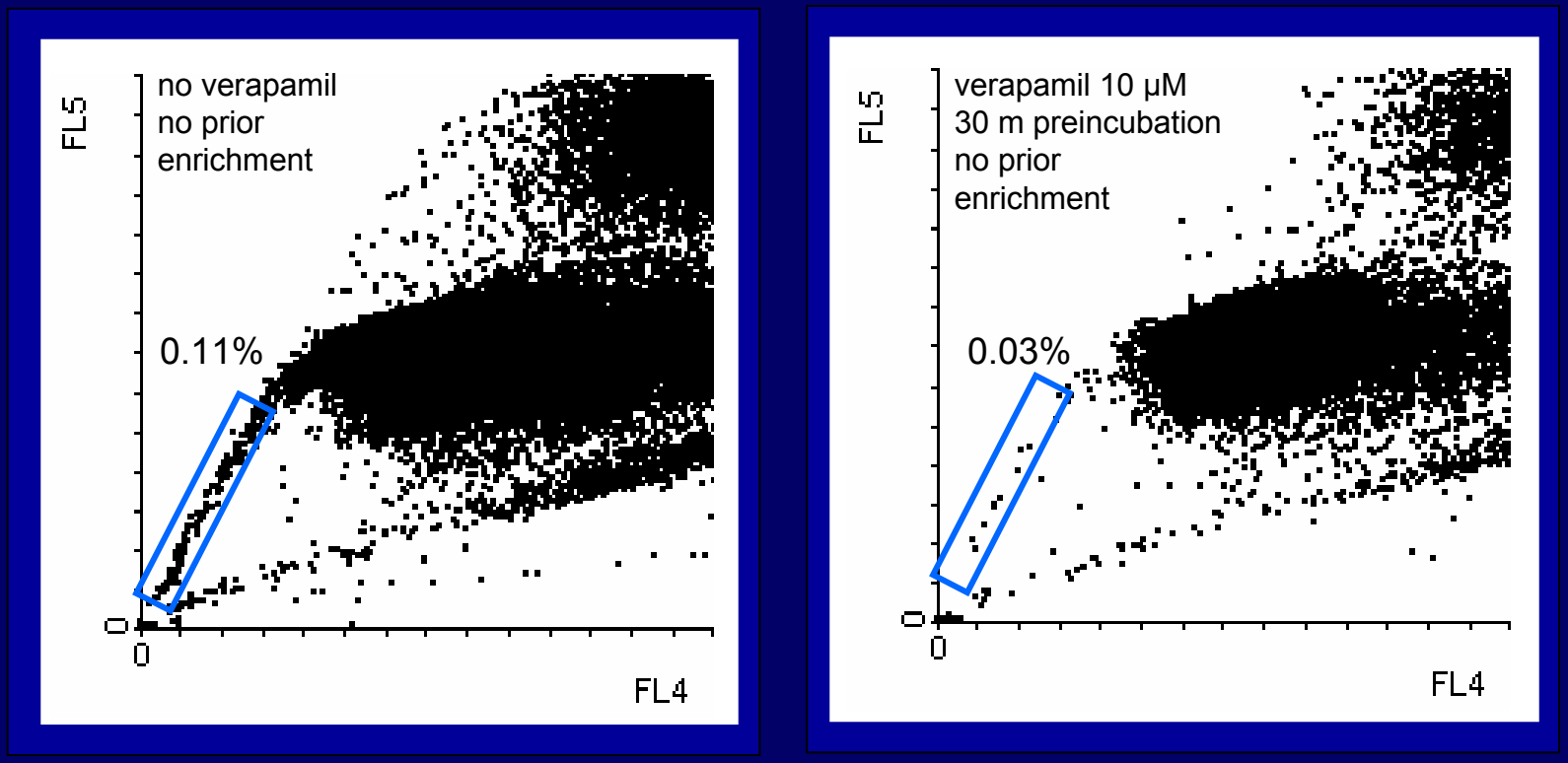


Discrimination of the Hoechst SP on the flow cytometer





Verapamil blocks the accumulation of Hoechst SP cells

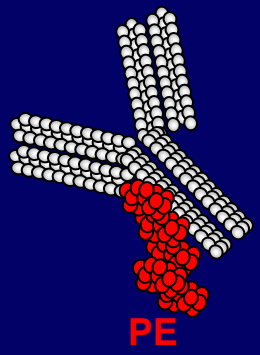


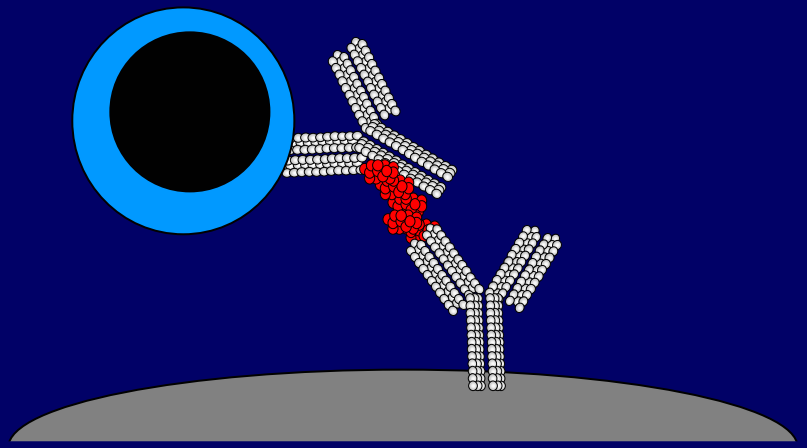
Samples from Drs. Atsushi Terasumi and John Vogel, DB, NCI, NIH

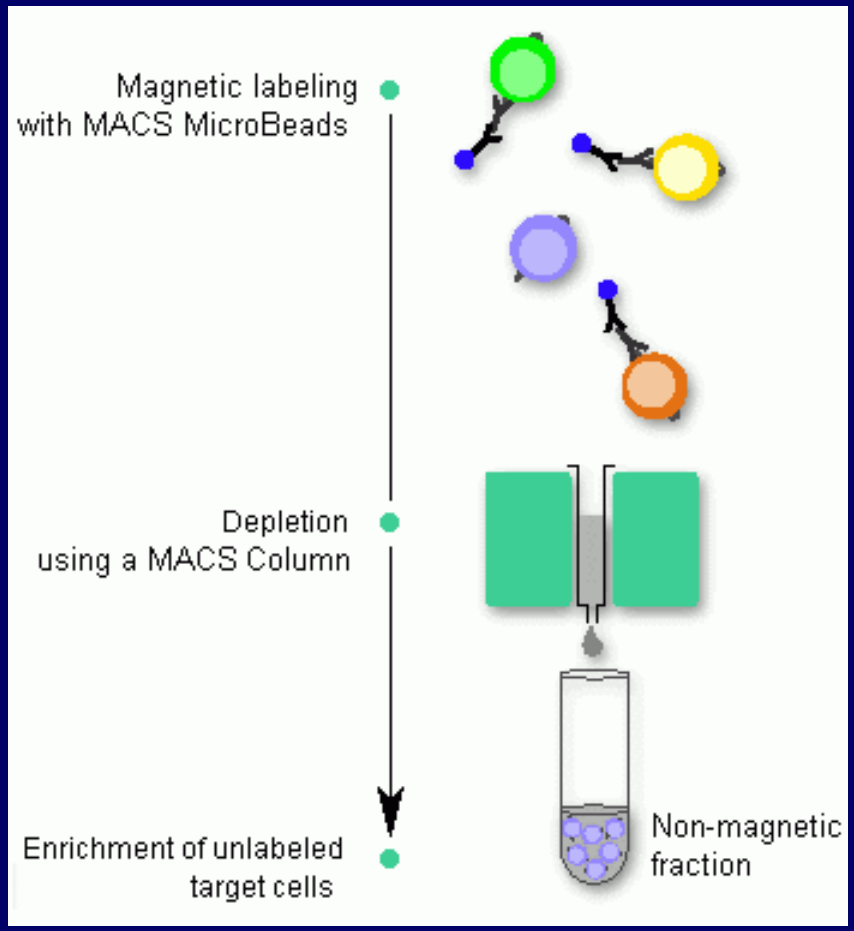
Negative selection of lineage-positive cells by magnetic bead depletion

Label bone marrow with hapten
or PE-conjugated antibodies
against lineage markers

CD3
B220
Ly6C + G
Gr-1
Ter119
NK1.1
CD5

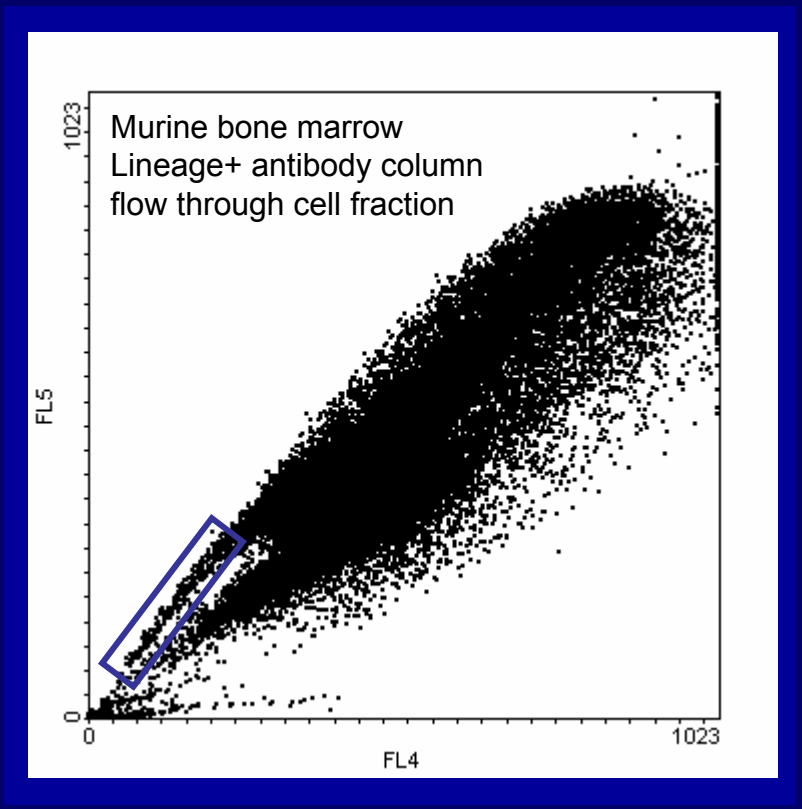


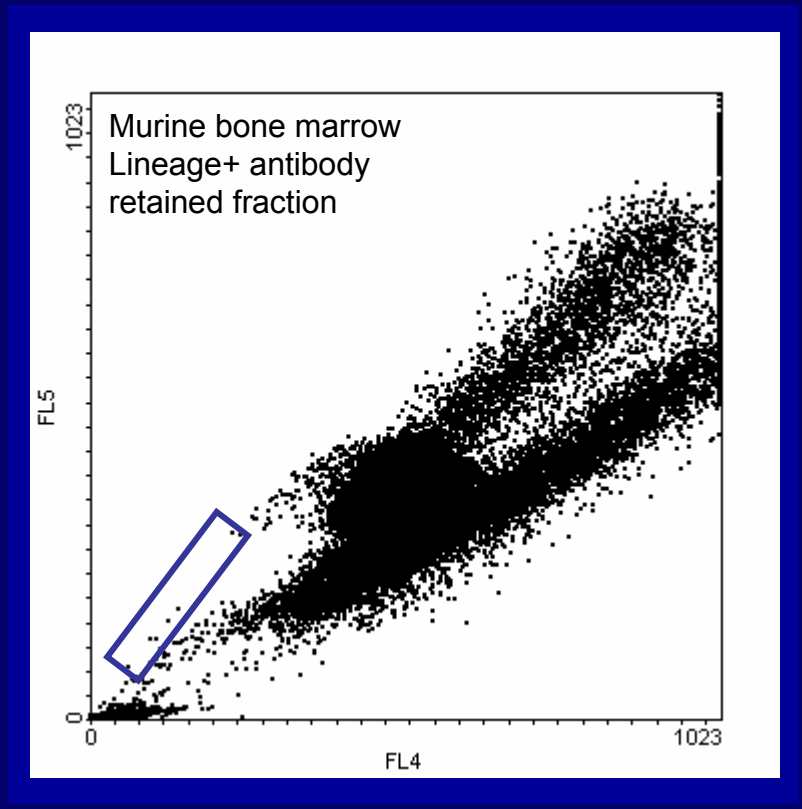




Discrimination of Hoechst SP on the flow cytometer

Lineage panel includes CD3, B220, CD11b, Ly6G, Ter-119





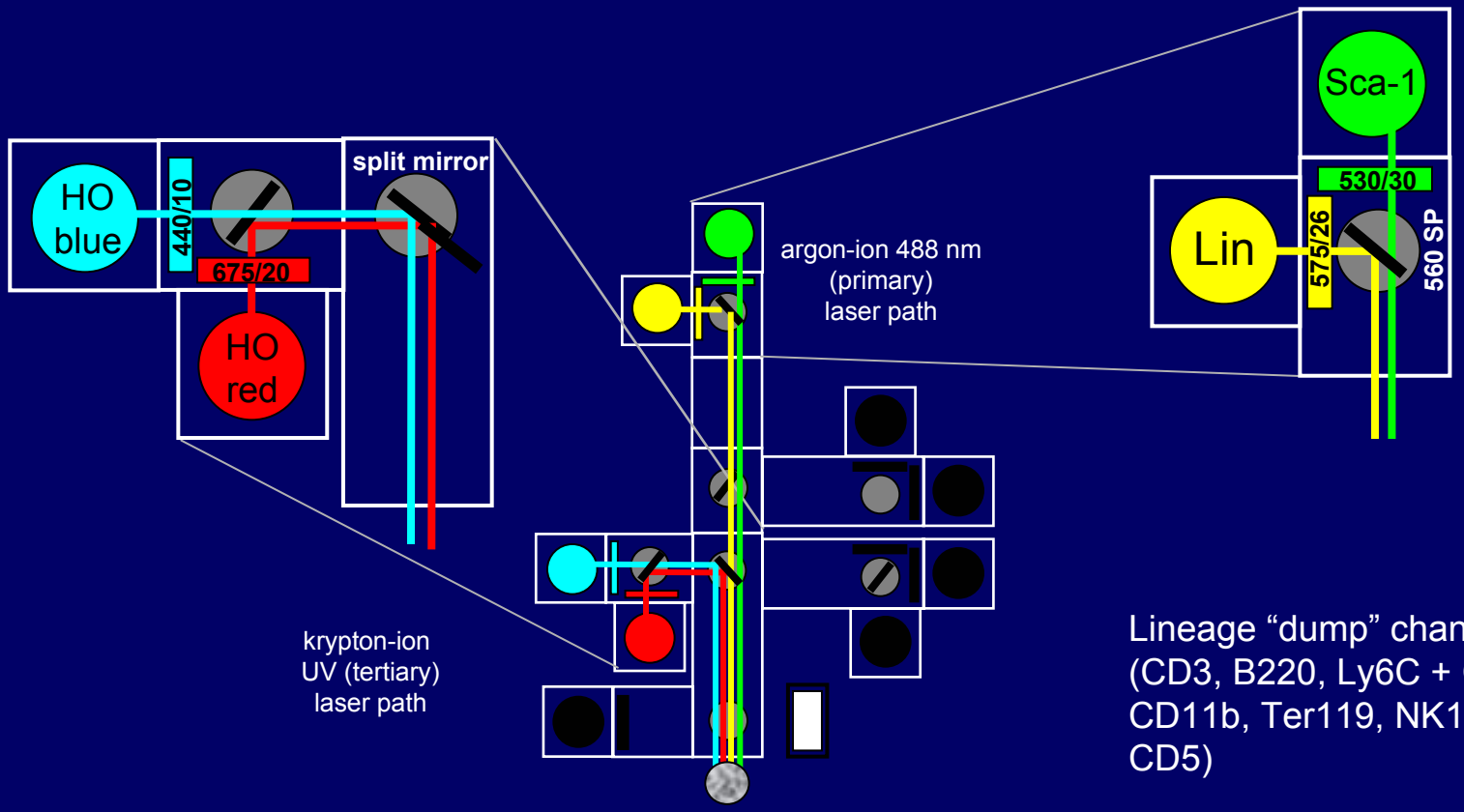
Hoechst 33342 Blue (440 nm)

Hoechst 33342 Red (675 nm)

Samples from Drs. Paul Love and Ella Frolova, NICHD, NIH

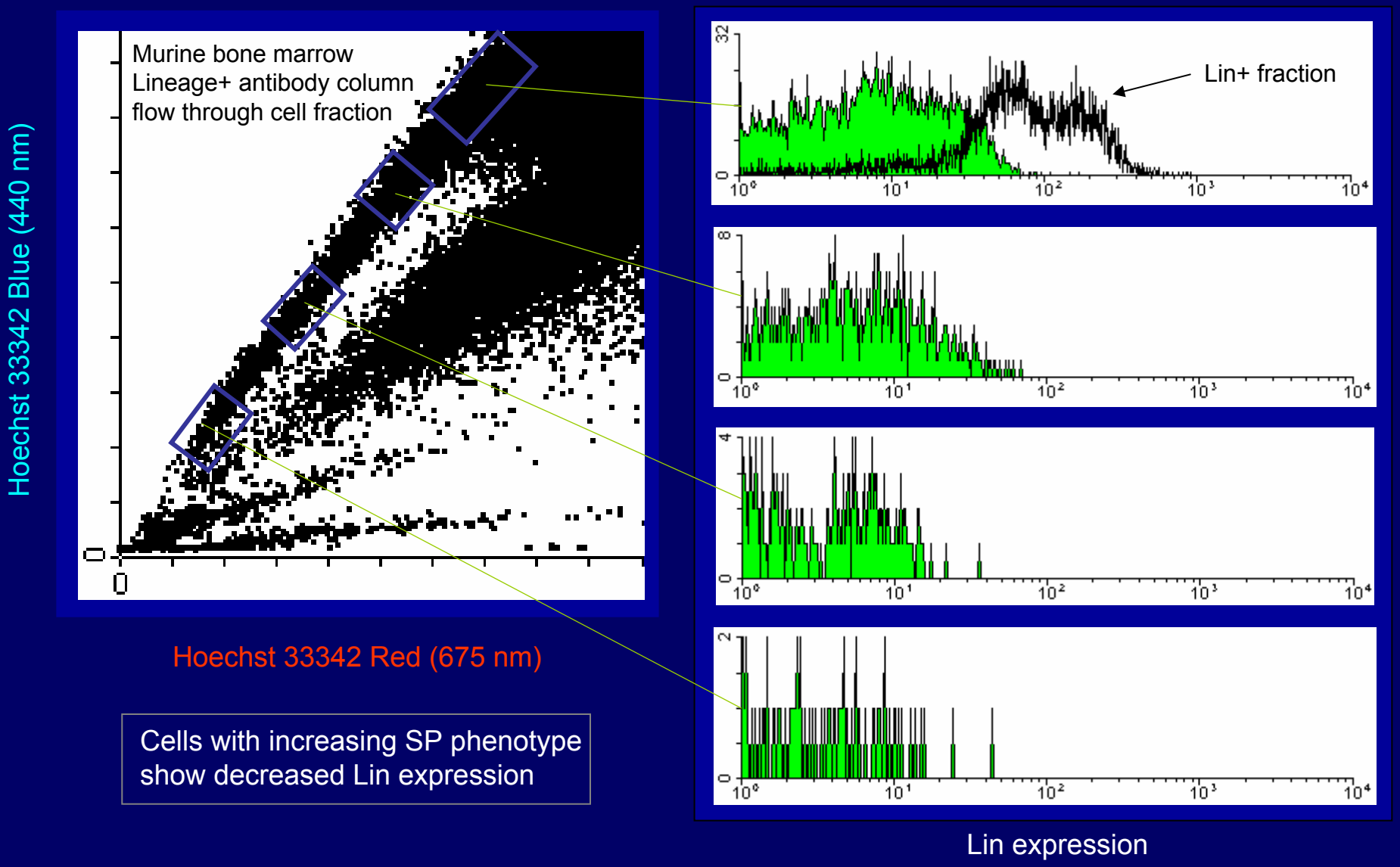
Discrimination of Hoechst SP on the flow cytometer

It is very possible (and highly desirable) to combine Hoechst SP analysis and cell surface immunophenotyping.



Lineage “dump” channel (CD3, B220, Ly6C + G, CD11b, Ter119, NK1.1, CD5)

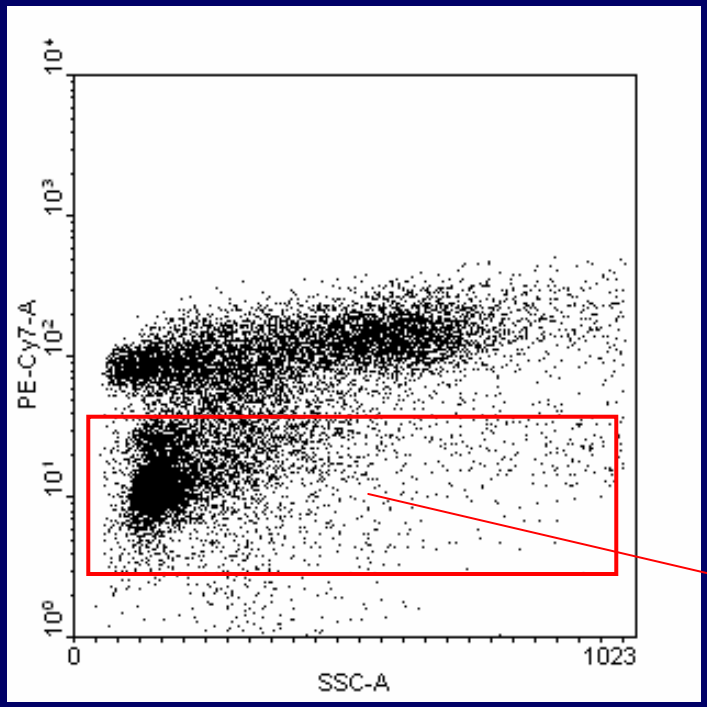
Lineage marker expression in Hoechst SP cells



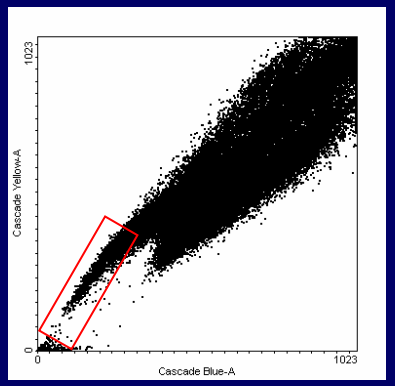
Cells with increasing SP phenotype show decreased Lin expression

Lineage exclusion and Hoechst SP analysis in mouse bone marrow

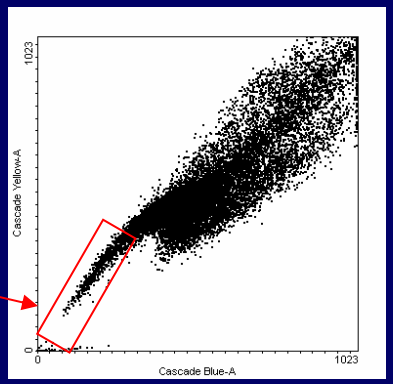
PE-Cy7 labeling for lineage panel (CD3, B220, Ly6C + G, CD11b, Ter119, NK1.1, CD5)



all cells

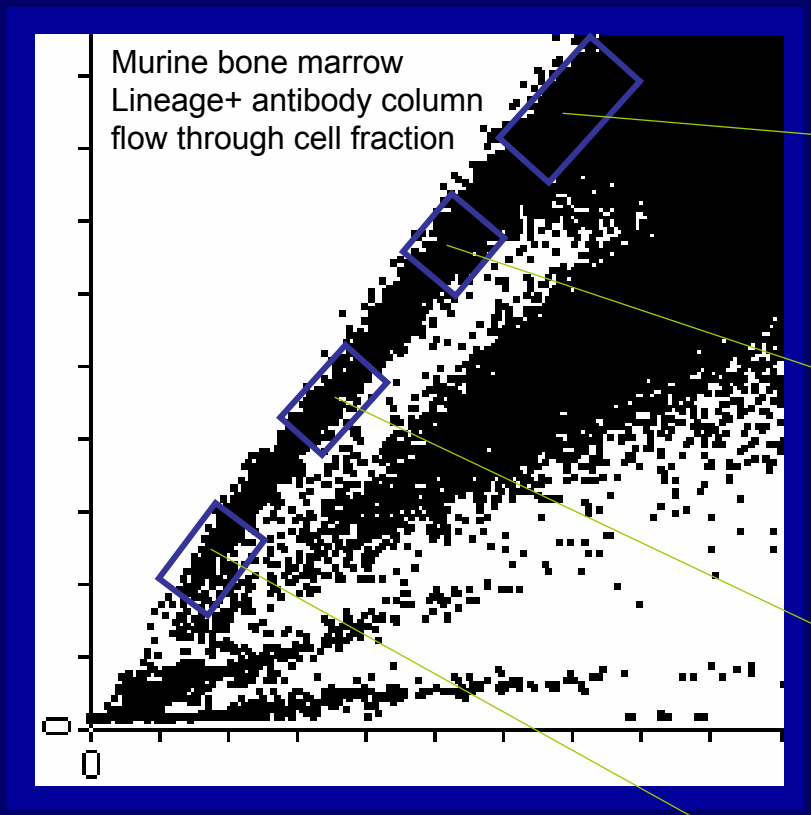


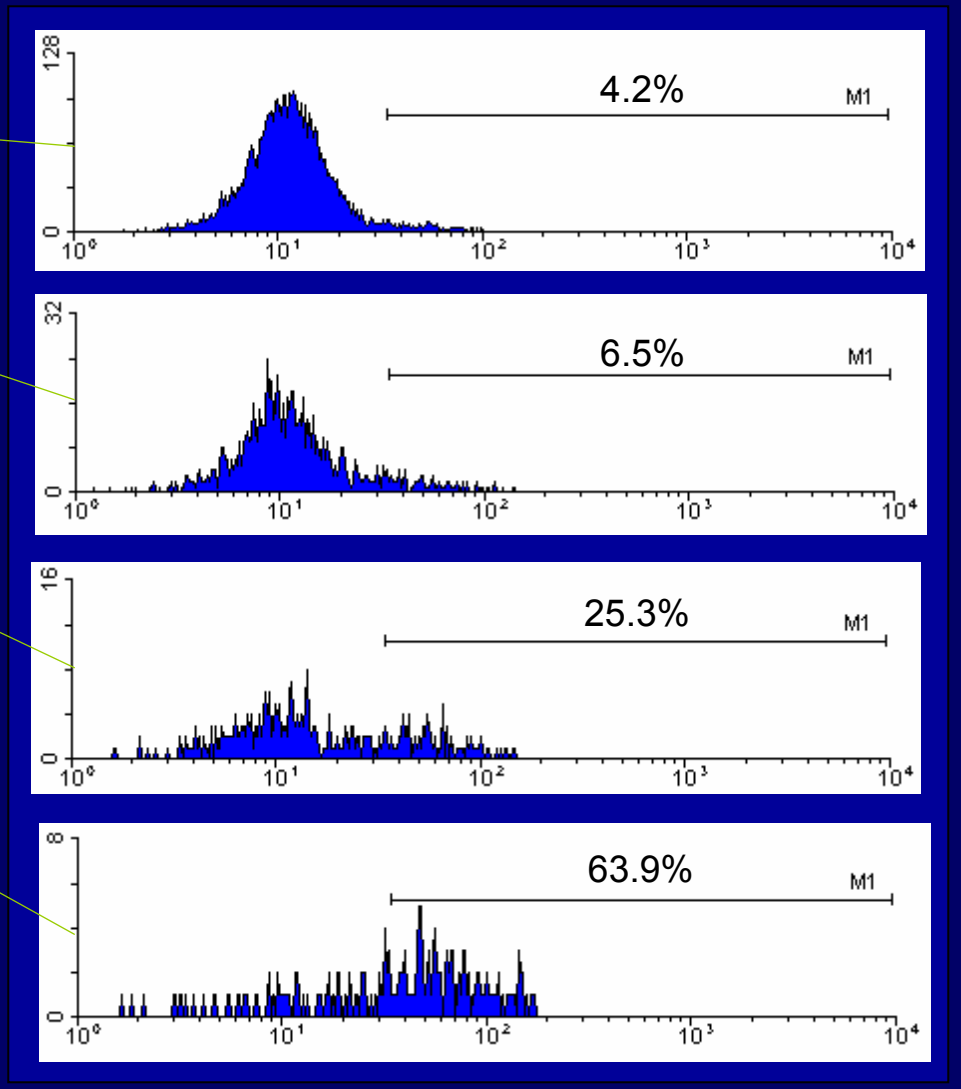
lineage negative



Lineage exclusion enriches for stem cells, but is insufficient alone for good isolation.

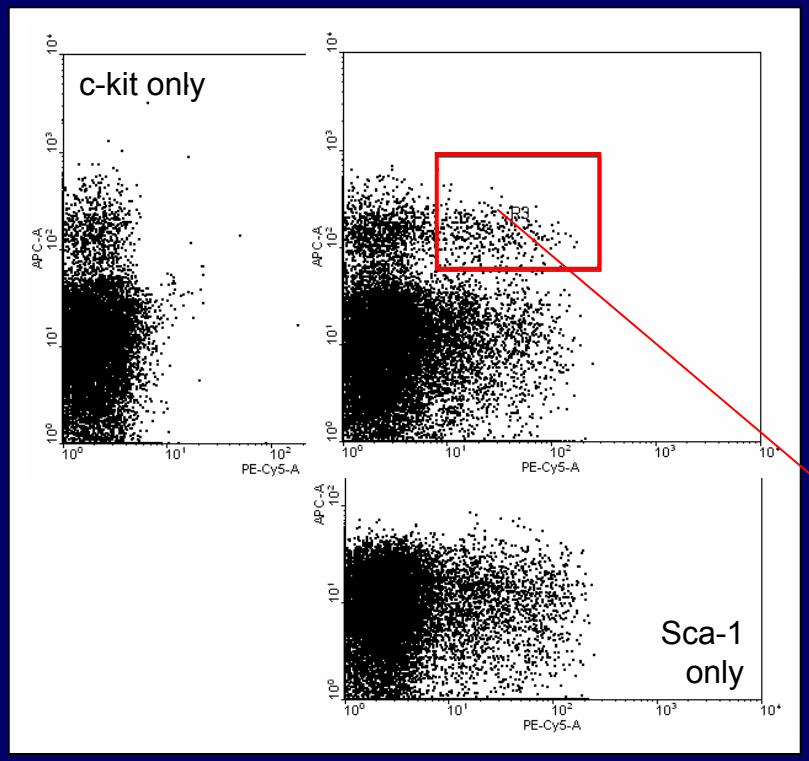
Sca-1 expression in SP subpopulation cells



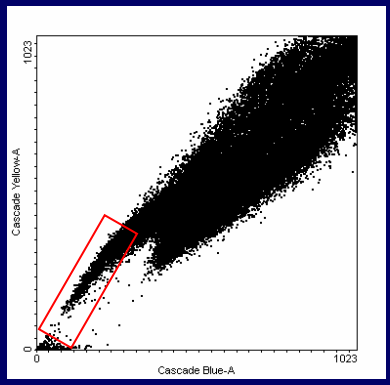


Cells with increasing SP phenotype
are enriched for Sca-1 expressing cells

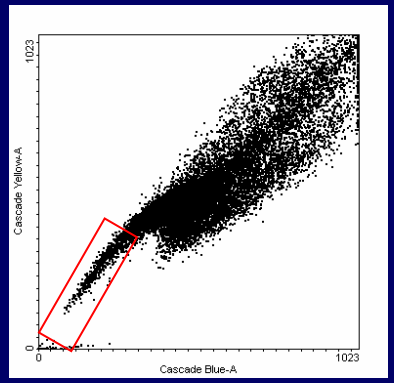
Lineage exclusion, Sca-1 and c-kit immunophenotyping in mouse bone marrow Hoechst SP analysis



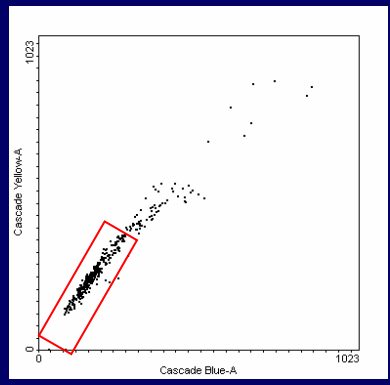
all cells



lineage negative



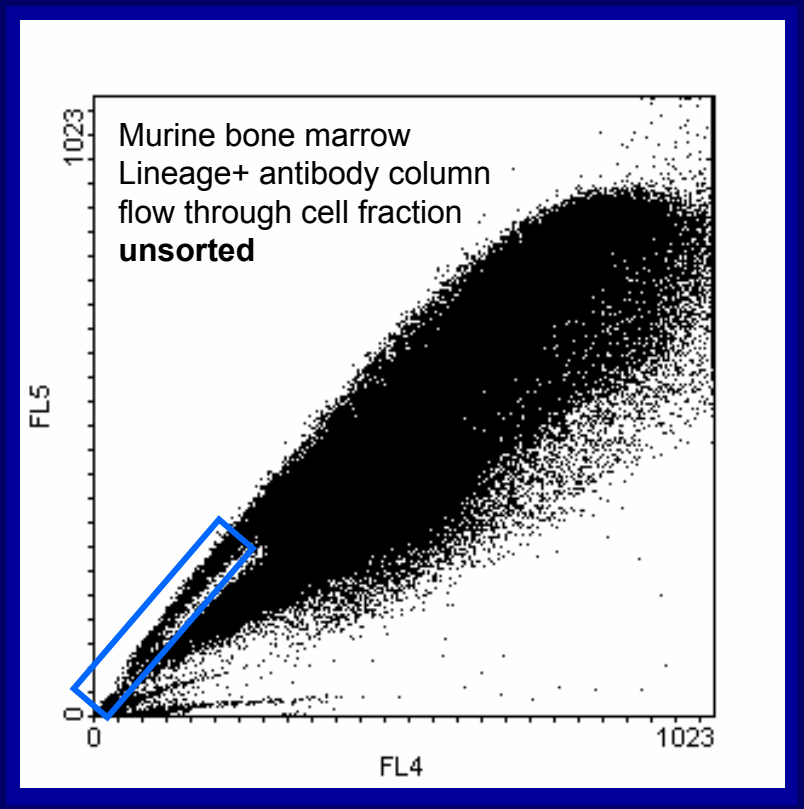
lineage negative Sca-1+ c-kit+

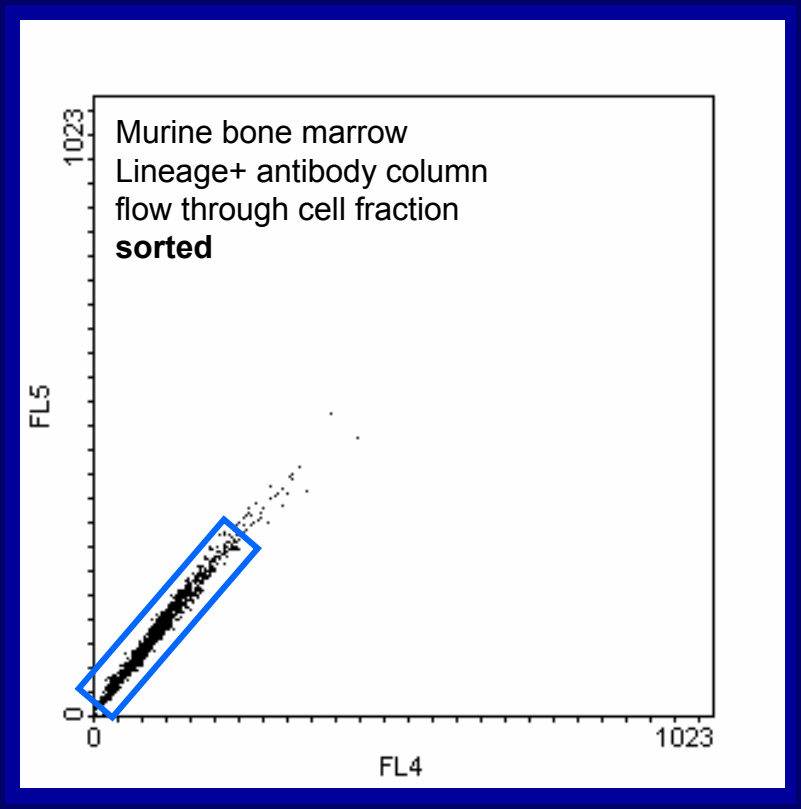


PE-Cy7 labeling for lineage panel (CD3, B220, Ly6C + G, CD11b, Ter119, NK1.1, CD5)

Sorting Hoechst SP cells

Lineage panel includes CD3, B220, CD11b, Ly6G, Ter-119





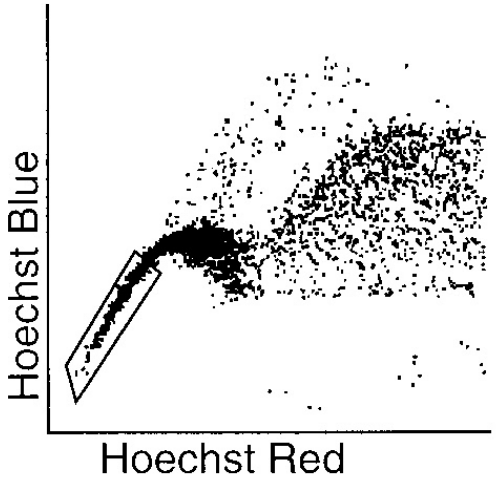
Hoechst 33342 Blue (440 nm)

Hoechst 33342 Red (675 nm)

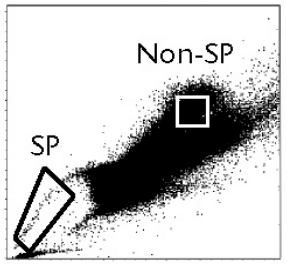
Tissue and species distribution of the Hoechst SP phenotype

A wide variety of stem cell types (hematopoetic and mesenchymal, embryonic and adult) from both non-primate and primate tissues exhibit some degree of ABC dependent SP activity.

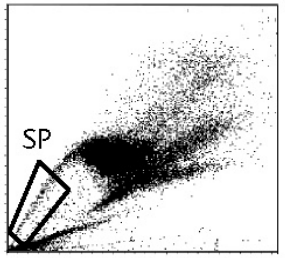
Mouse BM



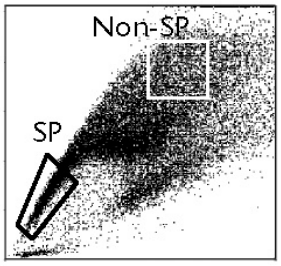
Monkey BM



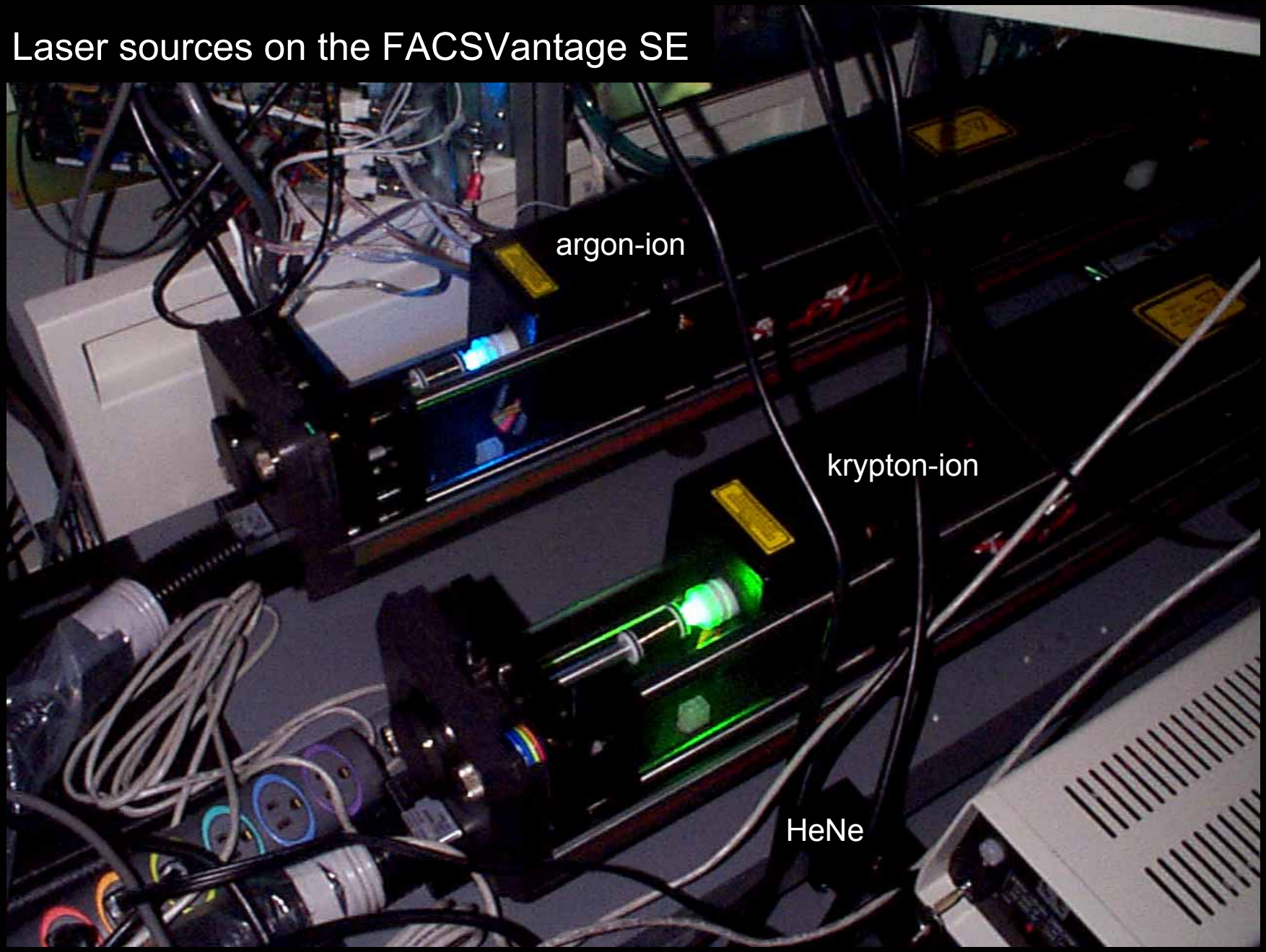
Mouse skeletal-muscle cells



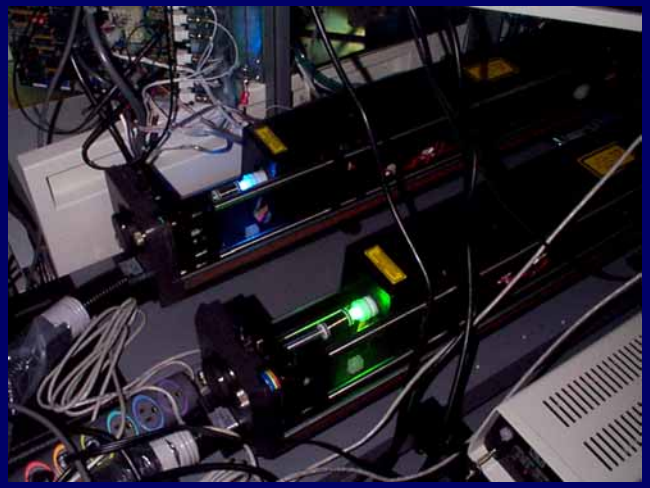
Mouse ES



Laser sources on the FACSVantage SE



Equipment required for analysis of Hoechst side population…

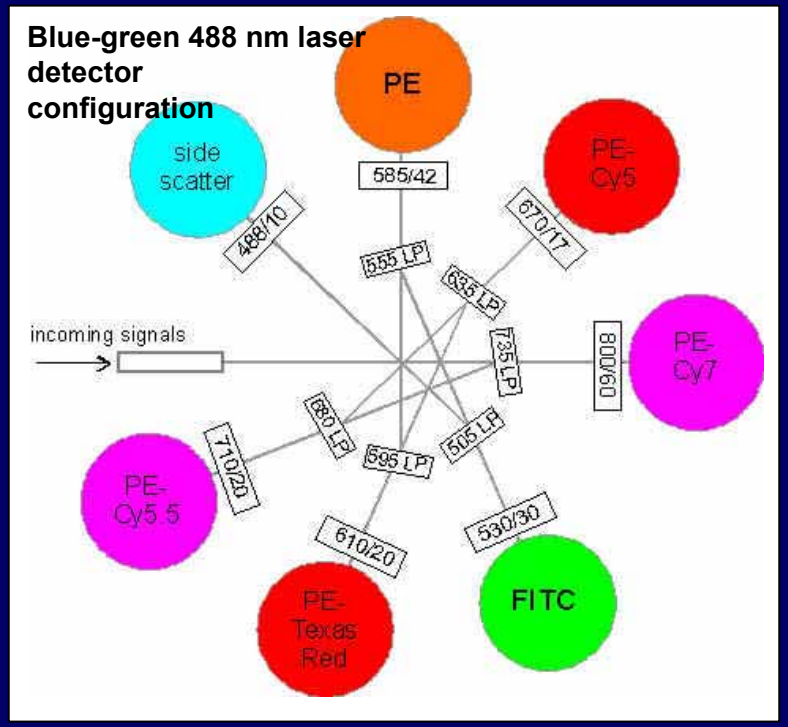


- Large scale cell sorter (i.e. FACSVantage DiVa, Beckman-Coulter Altra or Cytomation MoFlo
- High power argon-ion or krypton-ion laser **(US$ 30,000)**
- Total equipment cost = **US$ 400,000**

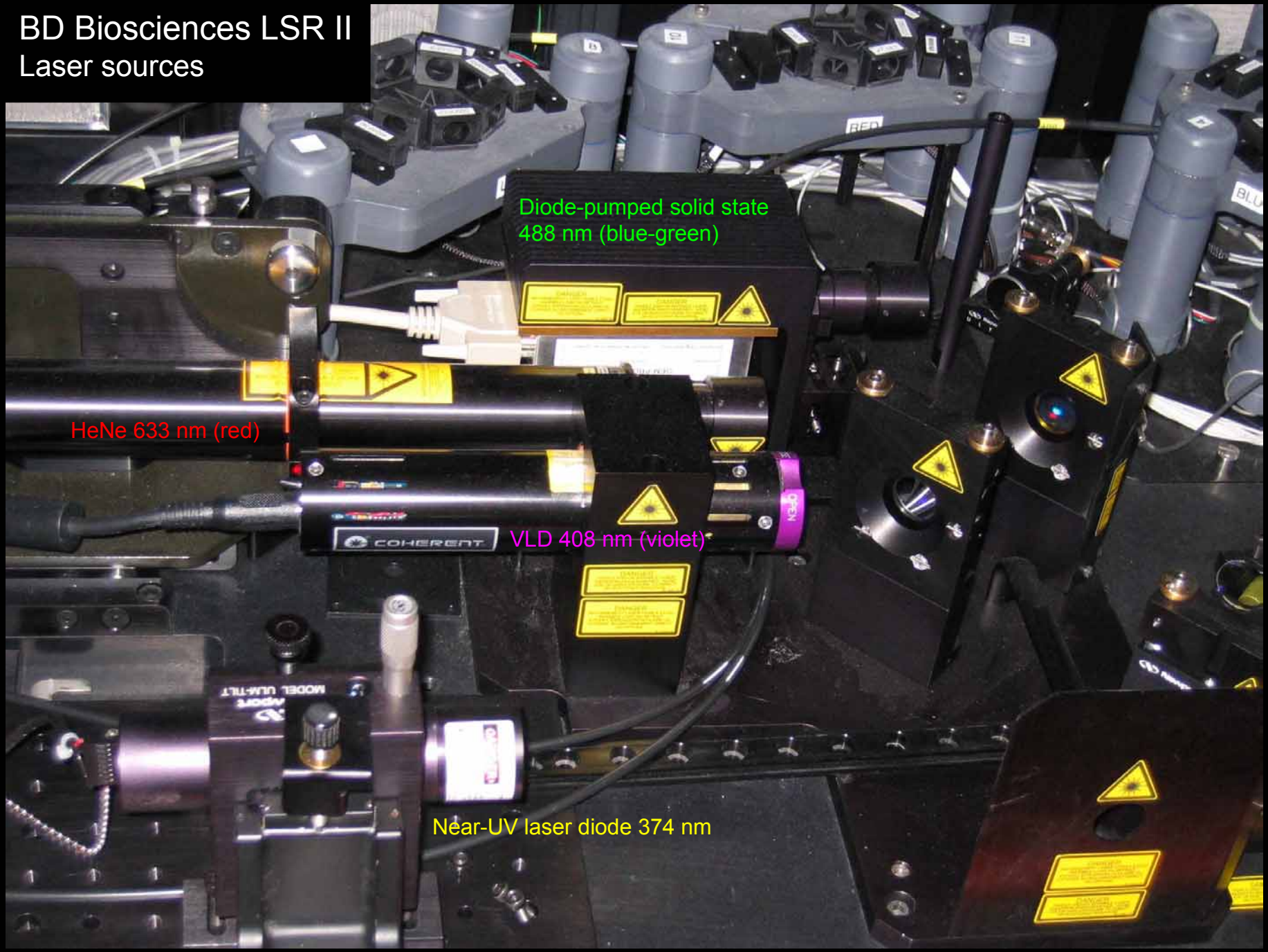
The equipment for analyzing Hoechst SP is prohibitively expensive for most institutions.

Polychromatic flow cytometers

- BD LSR II, Beckman-Coulter FC500, Cytomation CyAn
- Polychromatic cell sorting using a variety of laser sources
- Up to 10 colors simultaneously using up to four lasers



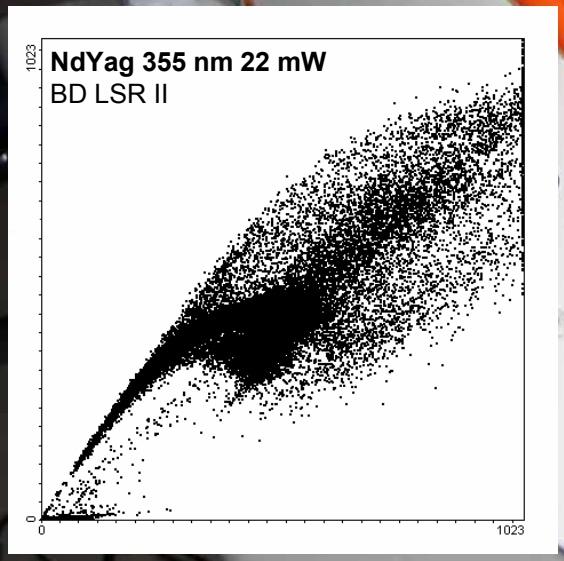
BD Biosciences LSR II
Laser sources



Novel laser sources for flow cytometry

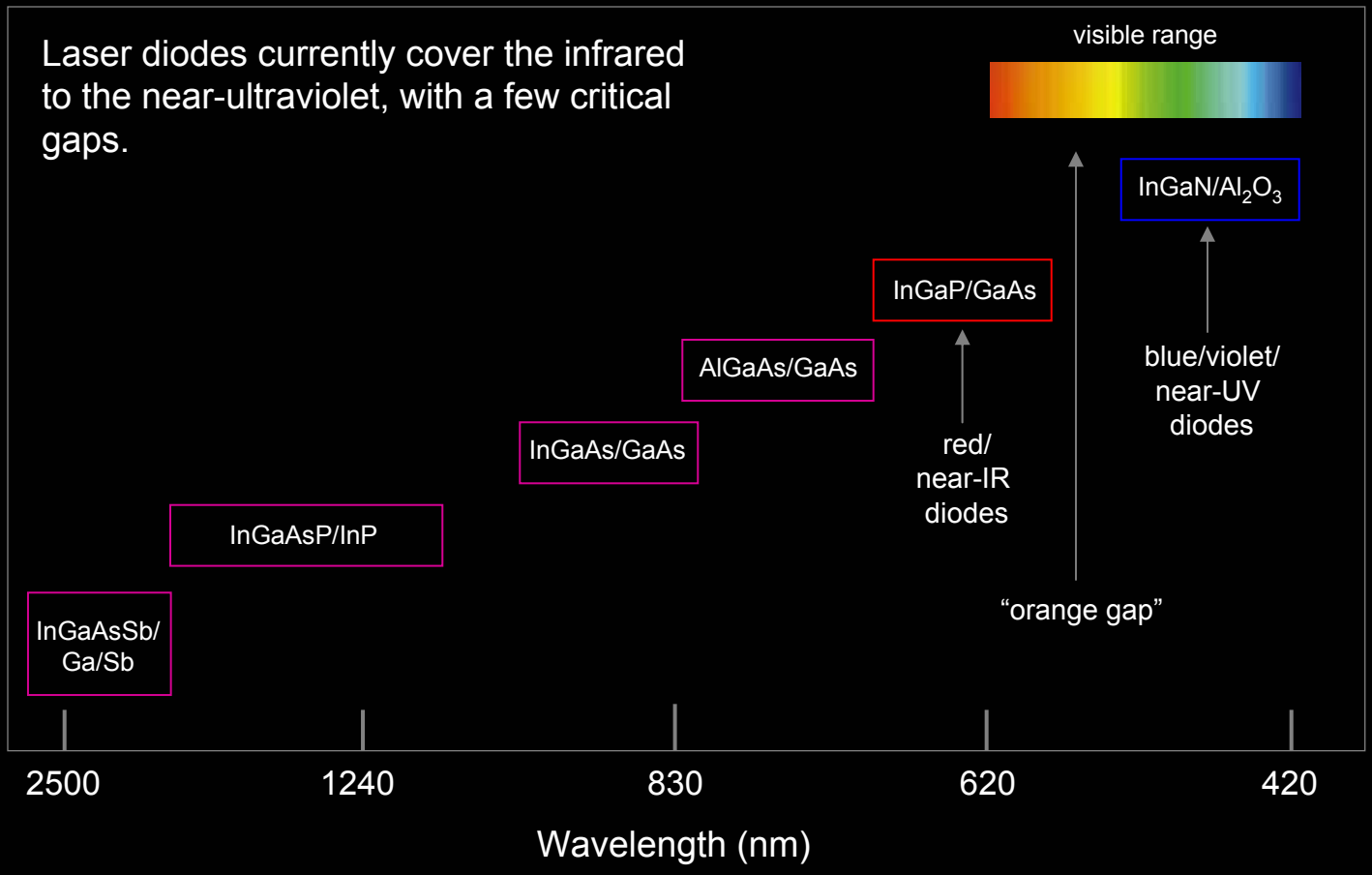
- **Laser diodes**
  - Near-infrared and red
  - Blue
  - Violet
  - **Near-ultraviolet**
- **Diode-pumped solid state**
  - Diode pumping of a solid state laser medium (such as yttrium aluminum garnet (YAG), or neodymium-YAG
  - Frequency doubling or tripling of can generate interesting laser lines for flow cytometry
  - DPSS green 532 nm
  - DPSS blue-green 460 – 490 nm
  - **Mode-locked Nd-YAG frequency-tripled 355 nm UV laser (quasi-CW)**

Lightwave mode-locked Nd-YAG 355 nm laser



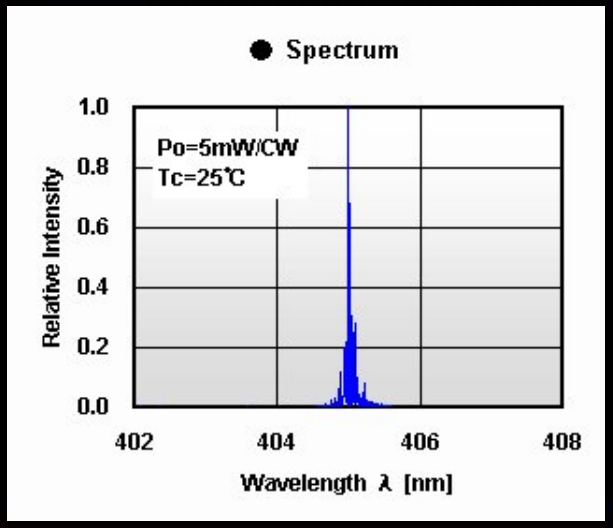
Cost is still high (about US$ 30,000)

Laser diodes



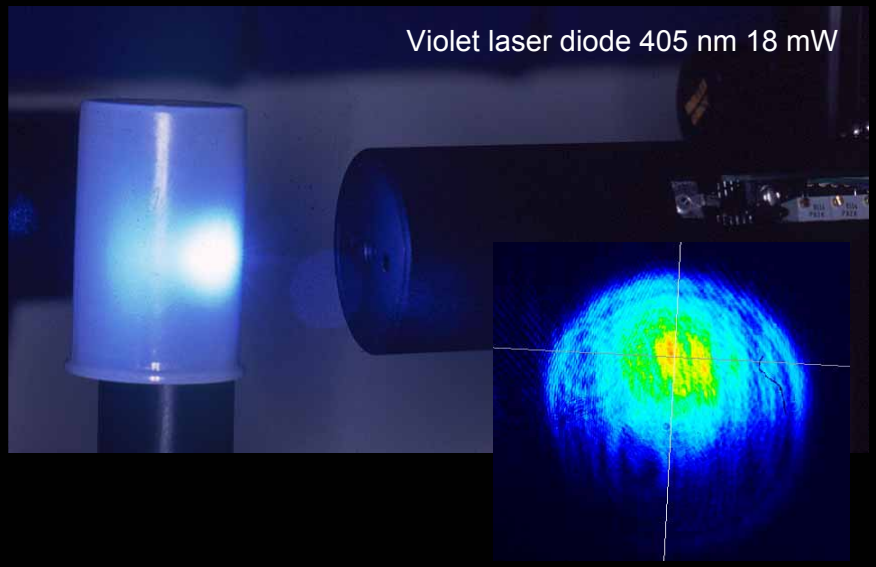
Single Transverse Mode Violet Laser Diode

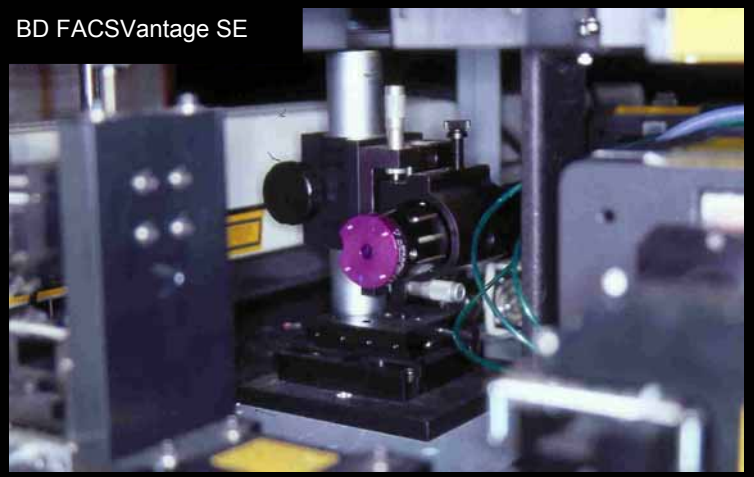
- can emit from 397 to 408 nm
- may provide a useful near UV laser source for both flow and laser scanning cytometry



Violet lasers on benchtop flow cytometers

- Solid-state violet laser diodes (VLDs) are now standard equipment on a wide variety of flow cytometers
  - BD LSR II and FACSAria
  - DakoCytomation CyAn
  - Compucyte LSC2 and iCys
- These small, reliable laser sources have broadened the use of violet-excited fluorochromes such as DAPI, Cascade Blue and Pacific Blue.

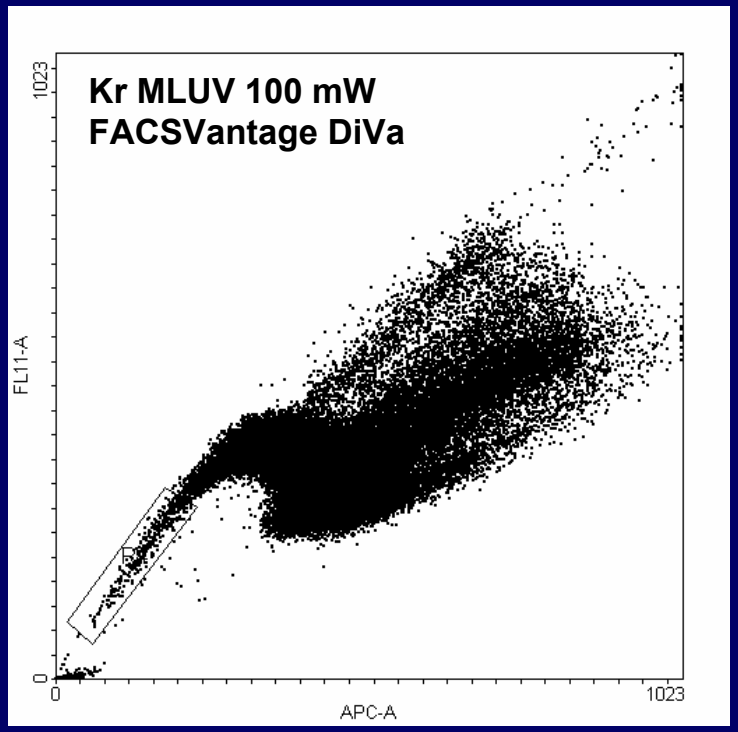


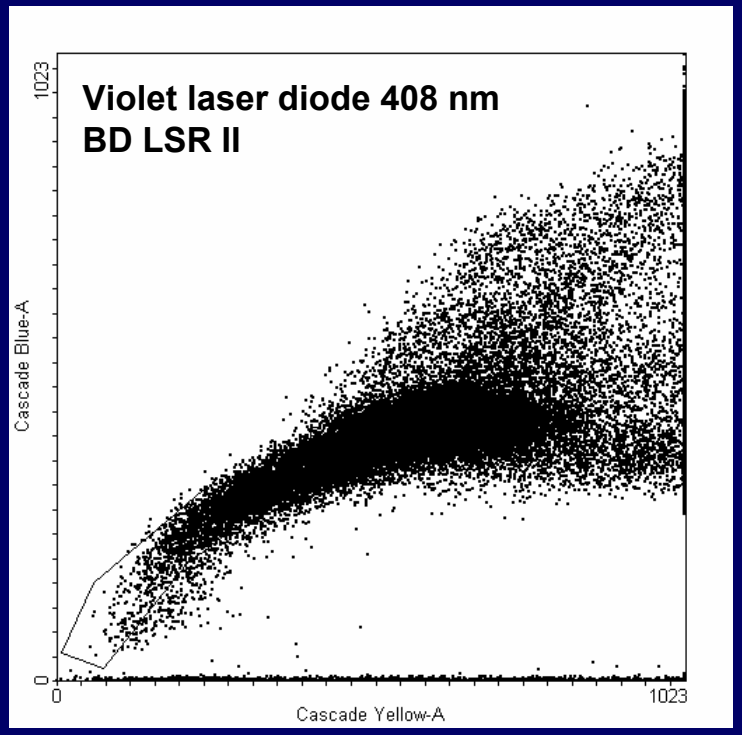


Hoechst SP Analysis using a Violet Laser Diode

Power Technology 408 nm 15 mW

Hoechst 33342 Blue (440 nm)



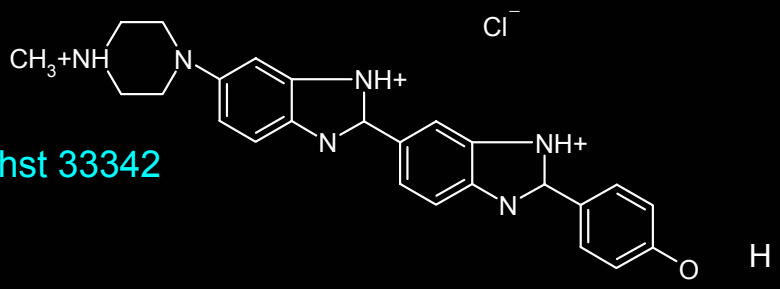


Hoechst 33342 Red (675 nm)

Murine bone marrow
No purification

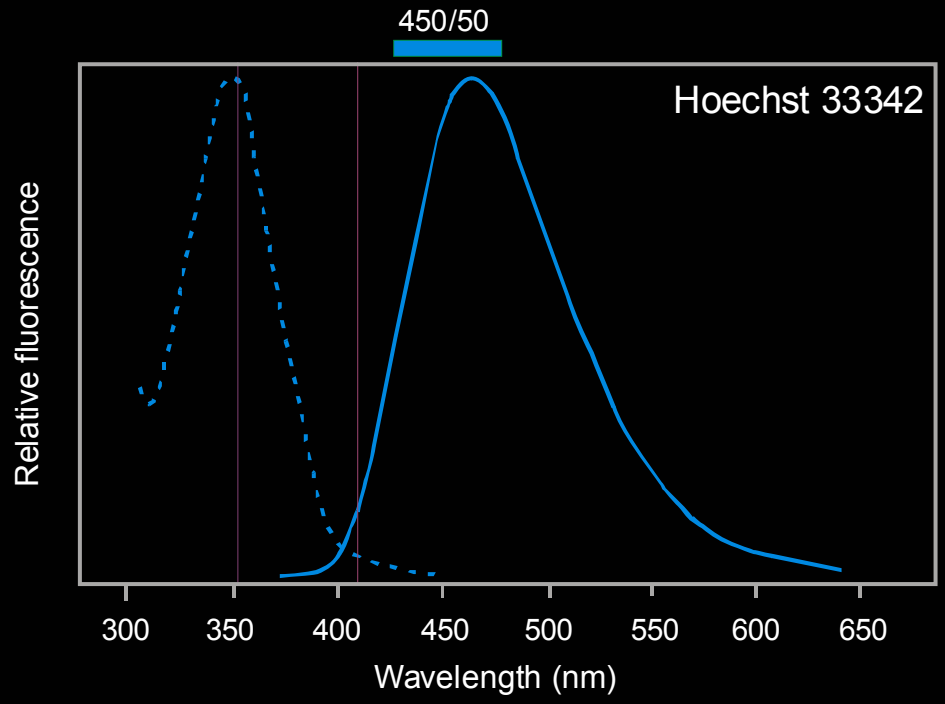
Violet laser diodes allow detection of the SP population,but with very low resolution.

Spectral Properties of Hoechst 33342



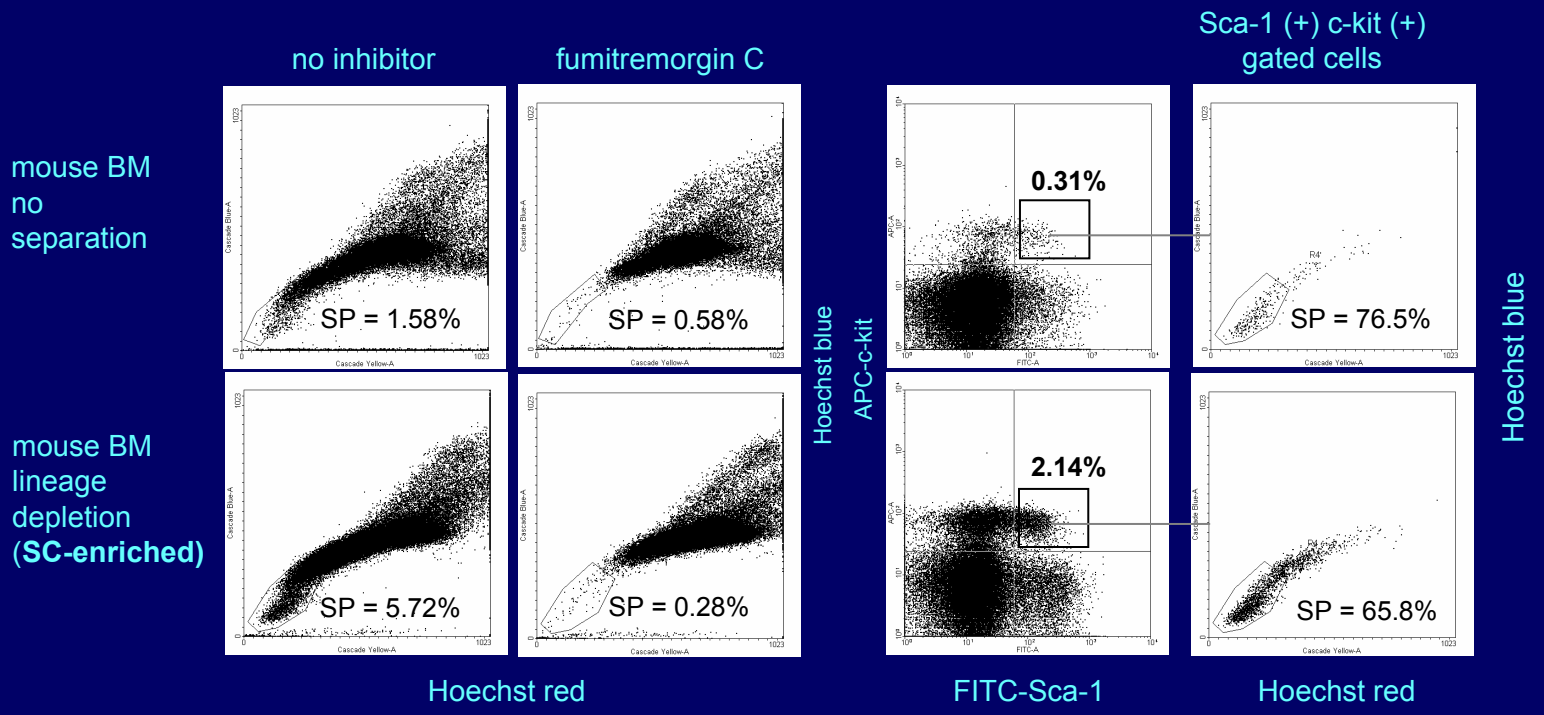
Hoechst 33342

The spectra of Hoechst 33342 suggests that it would be poorly excited by violet laser light.



Hoechst SP Analysis using a Violet Laser Diode

Violet diode 408 nm 25 mW



Violet laser diodes allow detection of the SP population,but with very low resolution.

Is good Hoechst SP resolution necessary?

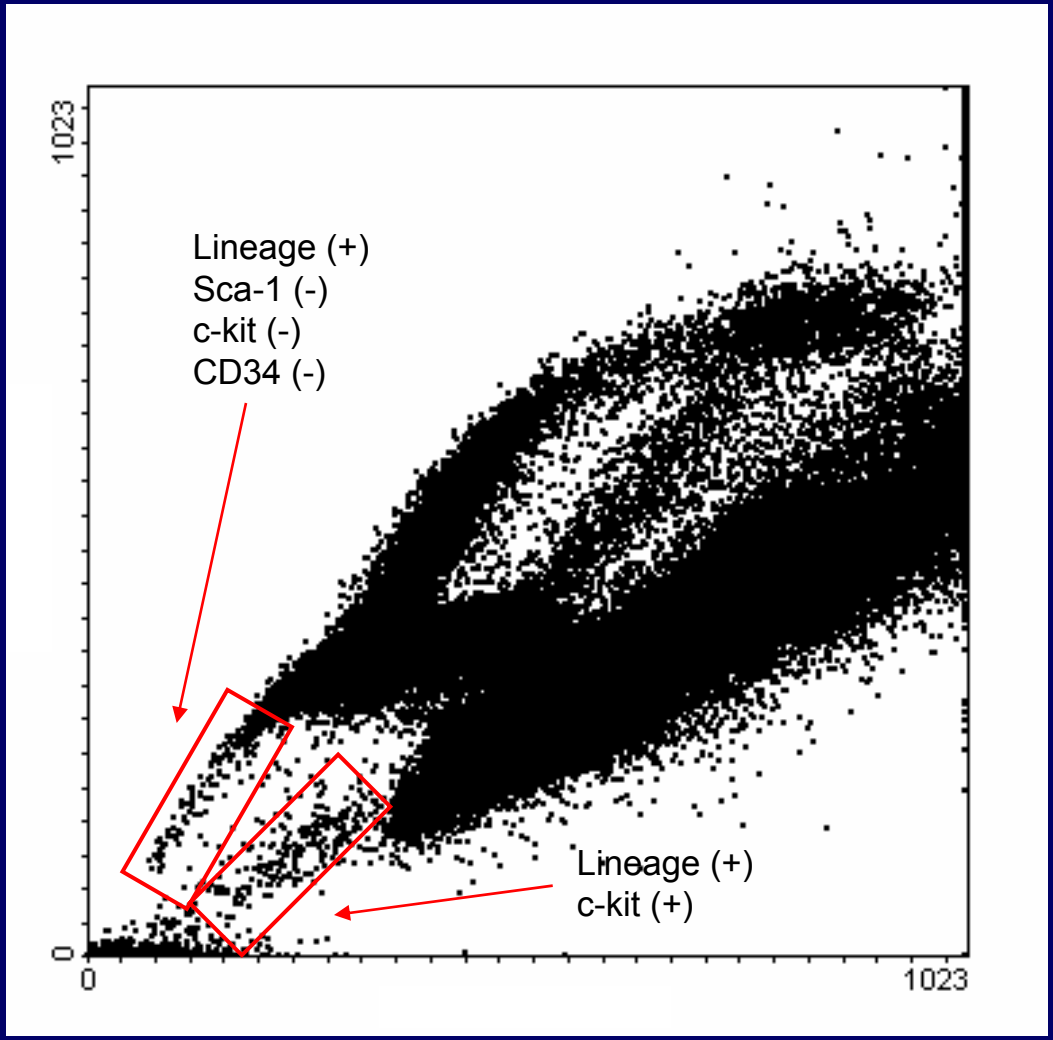
Yes, it is!

Hoechst 33342 labeled bone marrow often produces more than one hypodIploid population.

We aren't sure what these low-blue populations are, but they are NOT stem cells.

This is especially true of primate bone marrow and non-hematopoetic tissues.

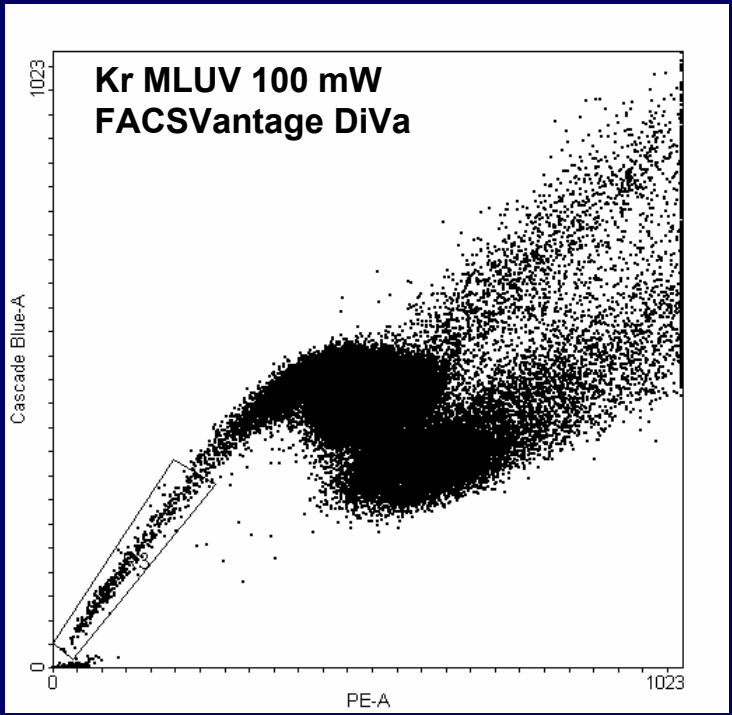
Suboptimal excitation of the Hoechst-labeled cells often fails to distinguish true SP cells from other non-stem SP populations.

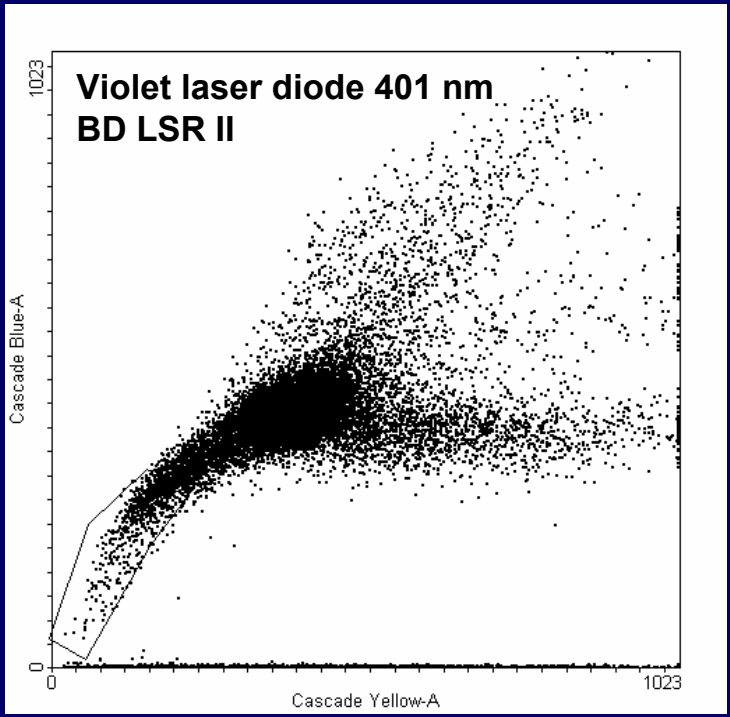


Hoechst SP Analysis using a Violet Laser Diode

Power Technology 401 nm 15 mW

Hoechst 33342 Blue (440 nm)





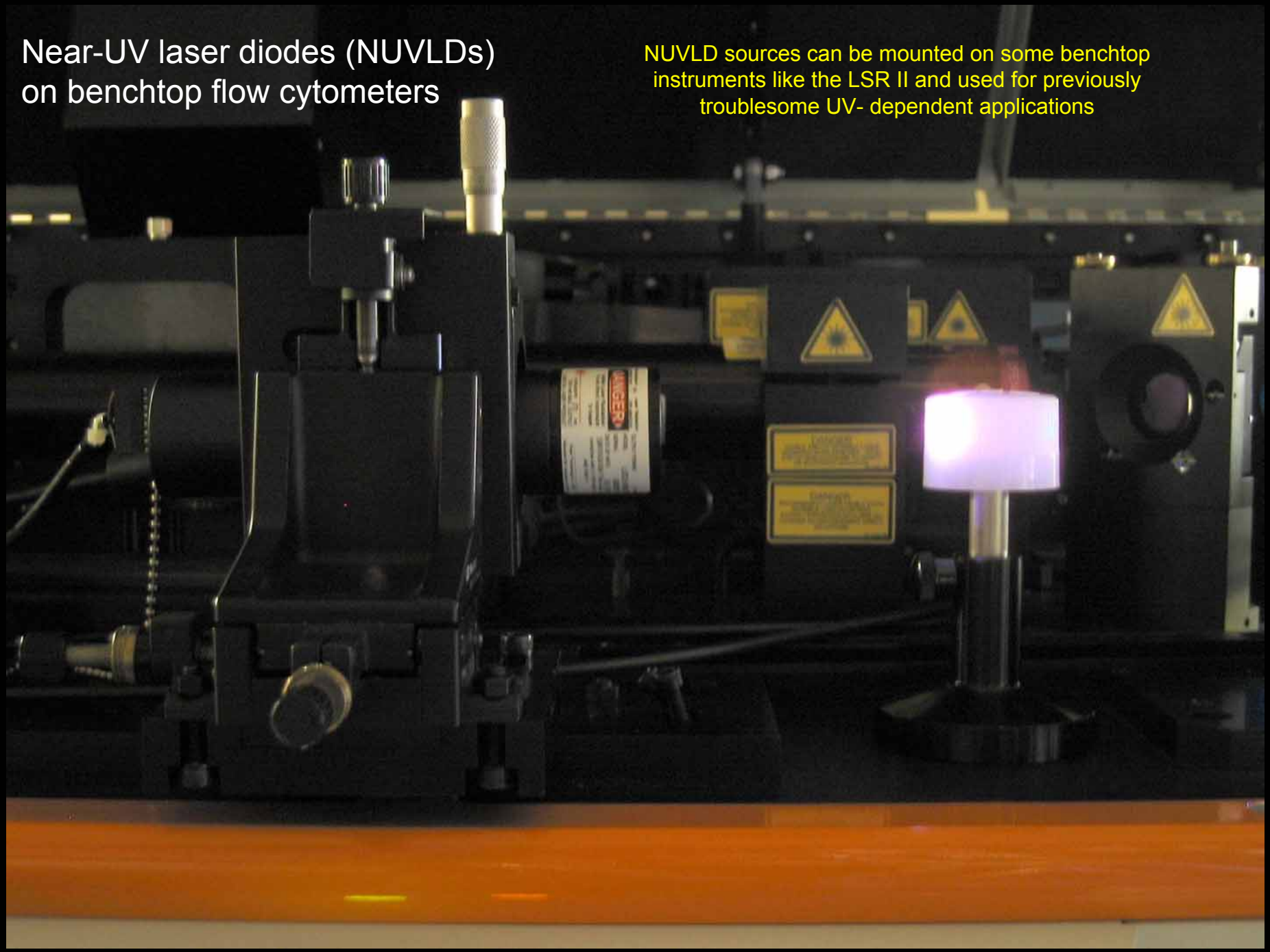
Hoechst 33342 Red (675 nm)

Murine bone marrow
No purification

Even short-wavelength violet diodes do not significantly improve SP resolution

Near-UV laser diodes (NUVLDs) on benchtop flow cytometers

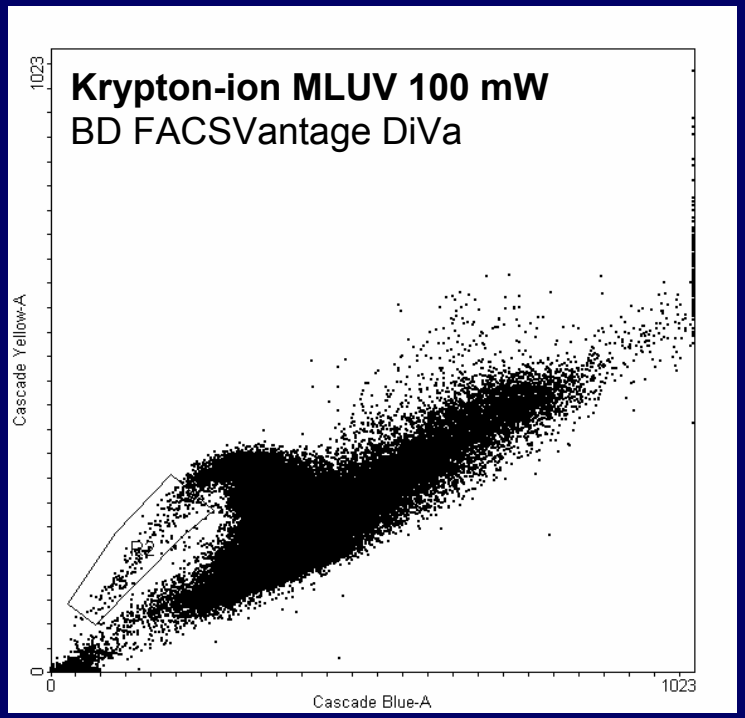
NUVLD sources can be mounted on some benchtop instruments like the LSR II and used for previously troublesome UV- dependent applications

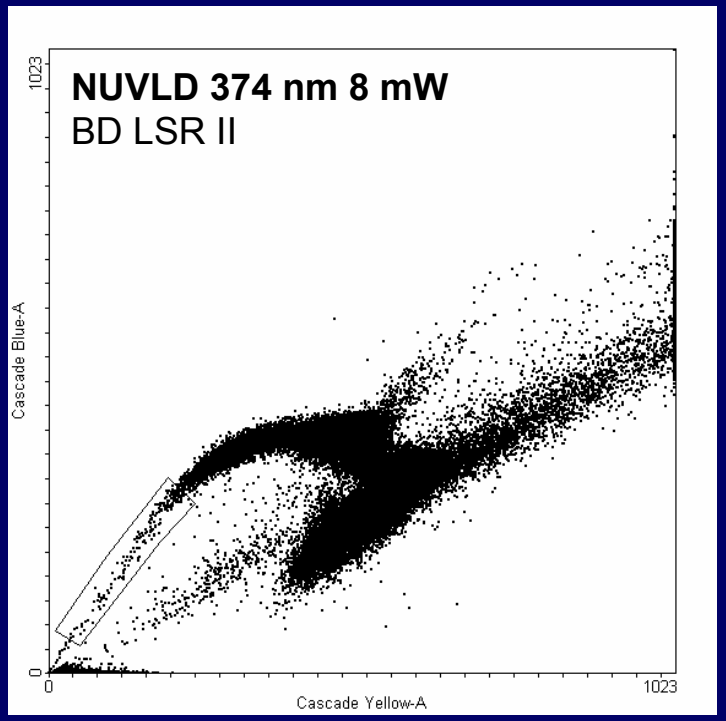


Near-UV laser diodes (NUVLDs) for Hoechst 33342 side population analysis

NUVLDs on cuvette instruments give better SP resolution than gas lasers on stream-in-air instruments.

Hoechst blue fluorescence (450/50)



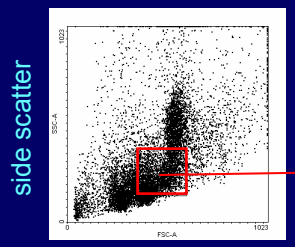


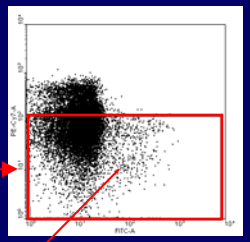
Hoechst red fluorescence (650 LP)

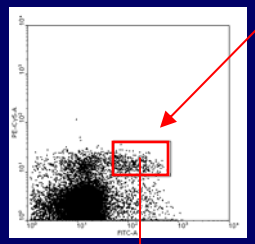
Six color bone marrow stem cell analysis

Requirements for simultaneous Hoechst SP analysis

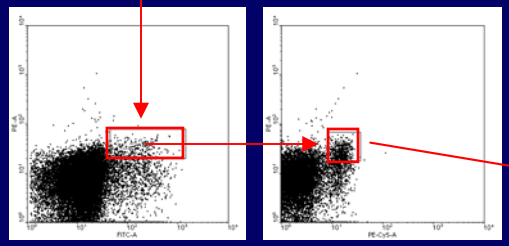
PE-Cy7 labeling for lineage panel
(CD3, B220, Ly6C + G, CD11b, Ter119, NK1.1, CD5)

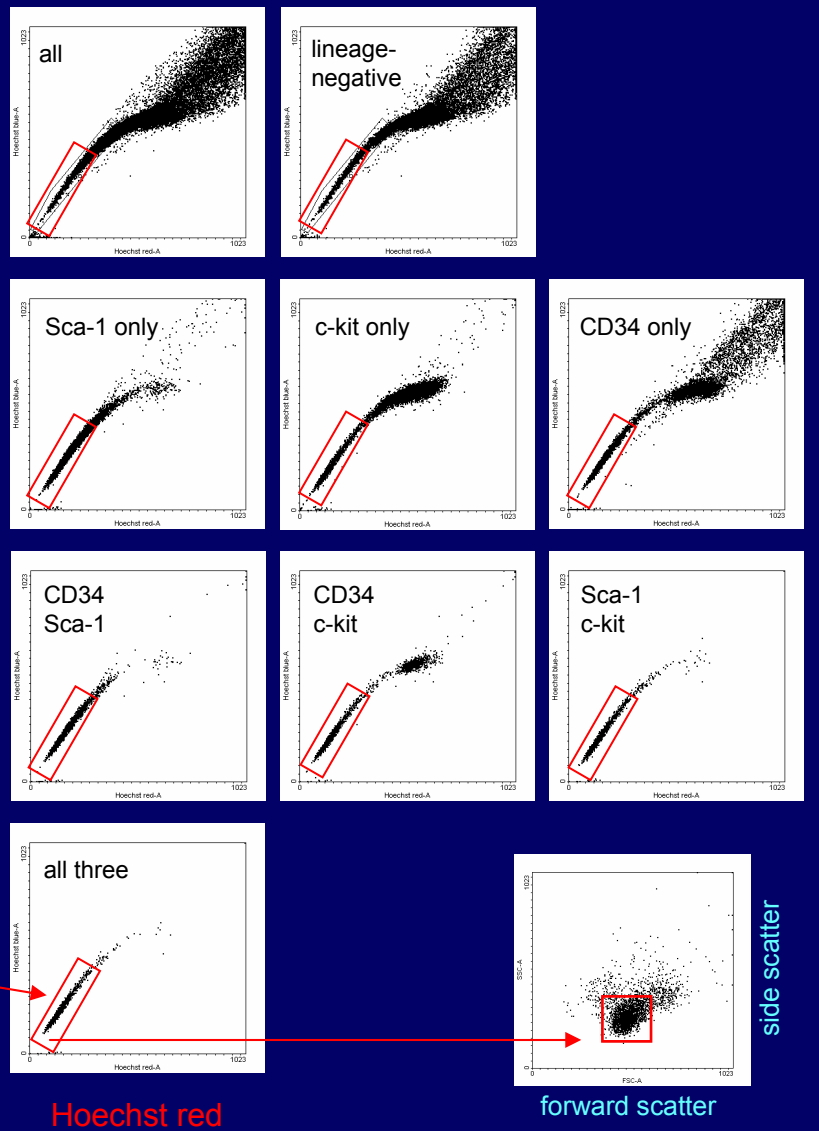






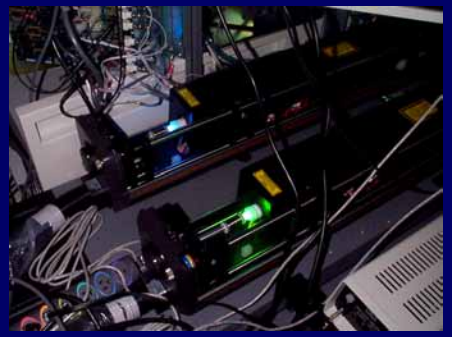
FITC-c-kit
PE-CD34
PE-Cy5.5-Sca-1
PE-Cy7-Lineage





Cost of producing a UV laser line for Hoechst SP analysis…

Krypton-ion multiline UV
361 - 365 nm laser
= **US$ 30,000**



Mode-locked Nd-YAG
355 nm laser
= **US$ 30,000**

Near-UV laser diode
375 nm laser
= **US$ 7,000**

Cost of instruments capable of doing Hoechst SP analysis…

FACSVantage with
Ar or Kr ion UV laser
= **US$ 400,000**

BD LSR II with
NUVLD or Nd-YAG laser

or

Cytomation CyAn
with Enterprise II laser
= **US$ 250,000**

Still pricey!

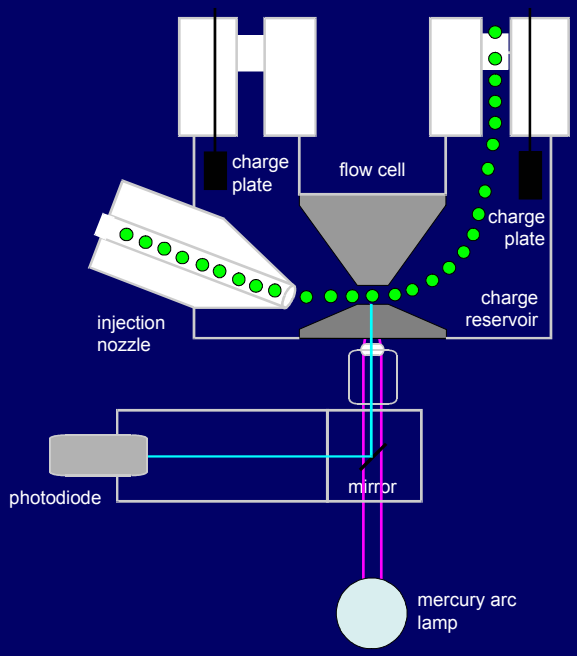
NPE Analyzer

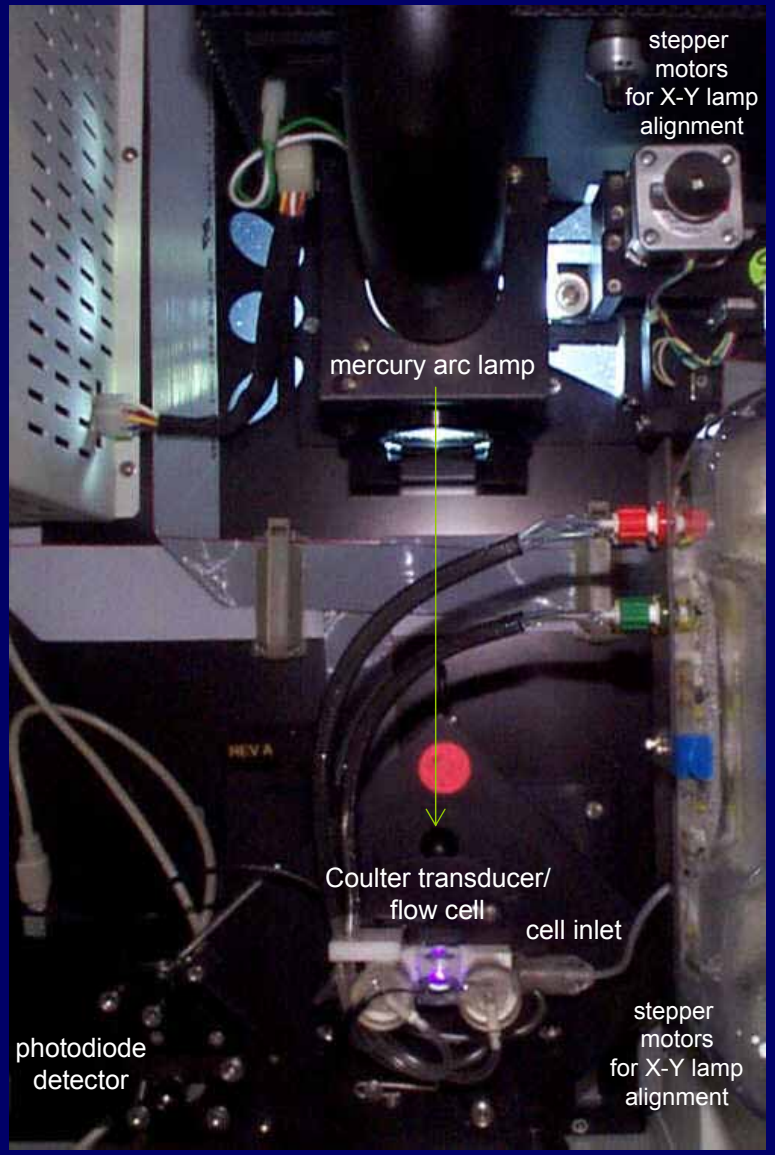
Next-generation flow cytometry technology still in the advanced development stage.

Utilizes a unique Coulter sizing transducer incorporated into a flow cytometry flow cell to allow both highly accurate electronic cell sizing and fluorescence analysis.

NPE Analyzer

Transducer flow cell assembly

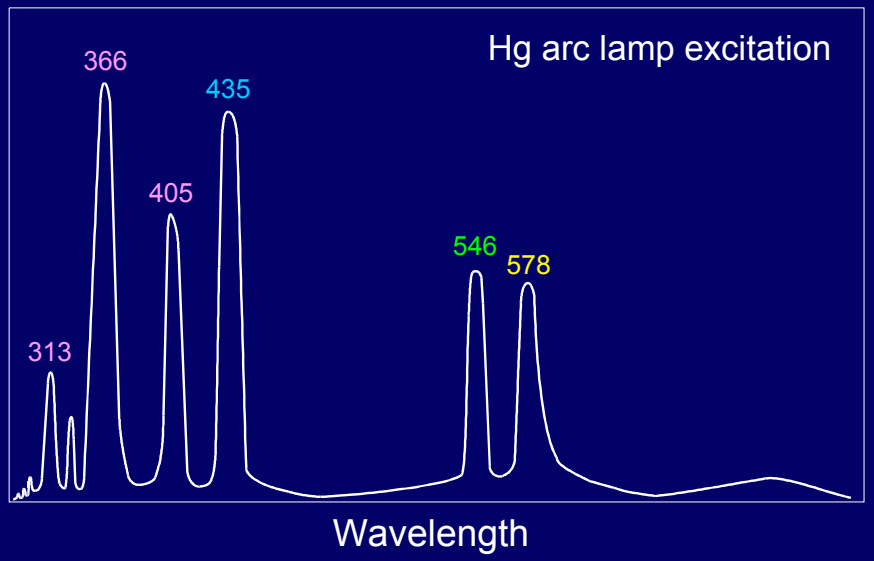




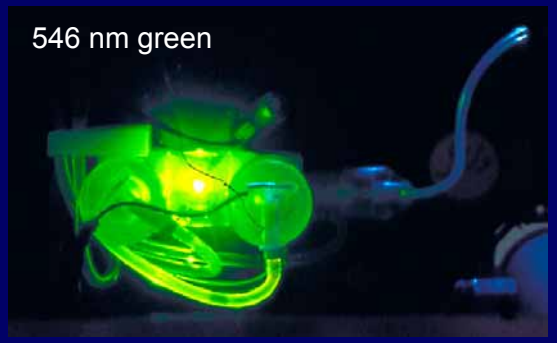
Can we use the NPE Analyzer to measure Hoechst SP?

Hg arc lamp derived UV excitation

We can “extract” most of the high-output lines from the Hg arc lamp for excitation of as variety of fluorochromes.

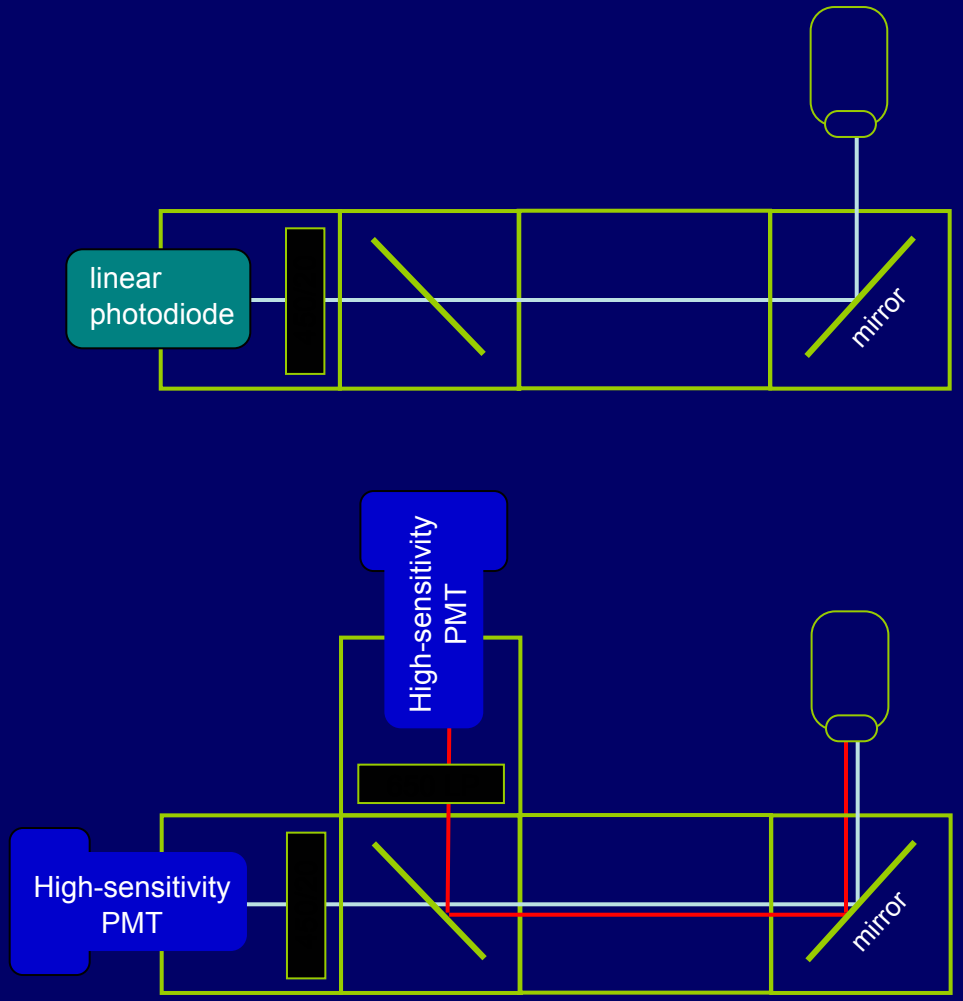


The Hg lamp emits a strong UV line at 365 nm.



Can we use the NPE Analyzer to measure Hoechst SP?
Optical layout

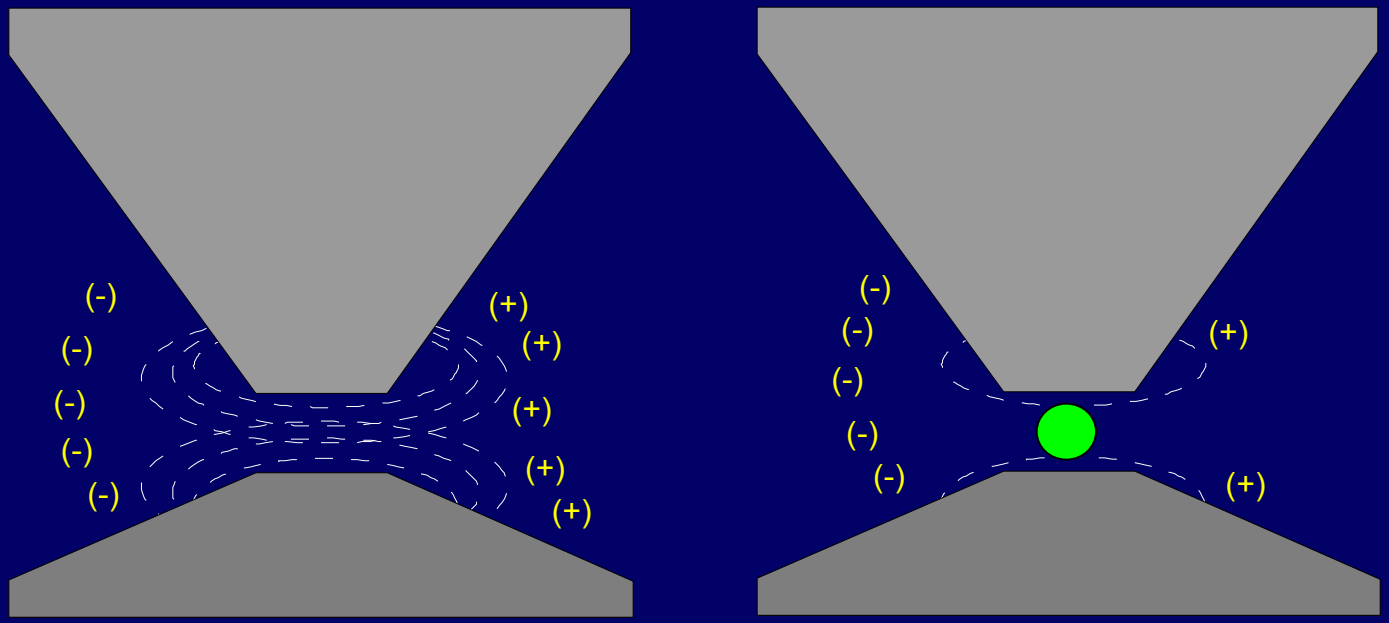
- Photodiodes have several unique advantages as optical detectors, are not as sensitive as traditional photomultiplier tubes
- NPE can be equipped with high-sensitivity photomultiplier tubes sensitive to as few as a few hundred photons per total detector area
- NPE set up a custom optical bench for us with two detector positions, both with PMTs.



Can we use the NPE Analyzer to measure Hoechst SP?

Electronic cell volume

The NPE Analyzer works on the same principle as the Coulter Counter, via the electrical resistance generated across an orifice by the occlusion of a particle.

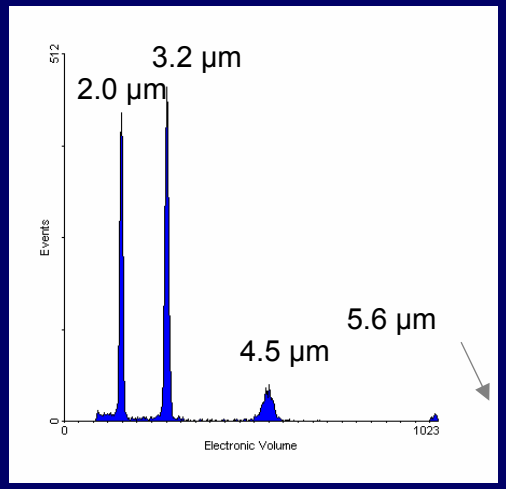


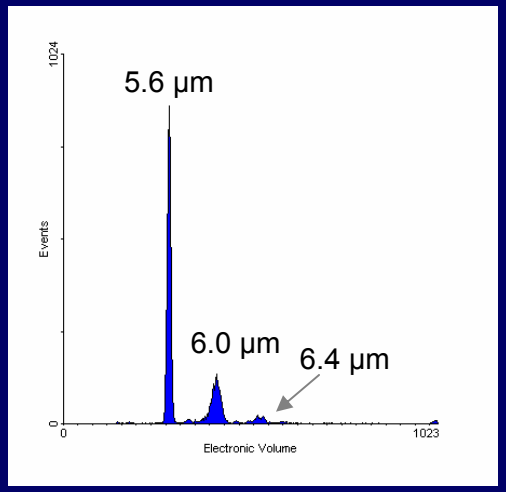
Coulter sizing provides a far more accurate measurement of particle size than traditional forward scatter measurement by flow cytometry

Can we use the NPE Analyzer to measure Hoechst SP?

Electronic volume measurement

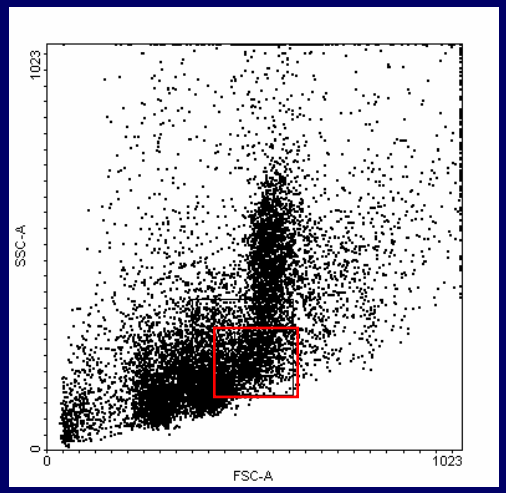
The NPE Analyzer can Measure electronic cell volume with a high degree of precision.





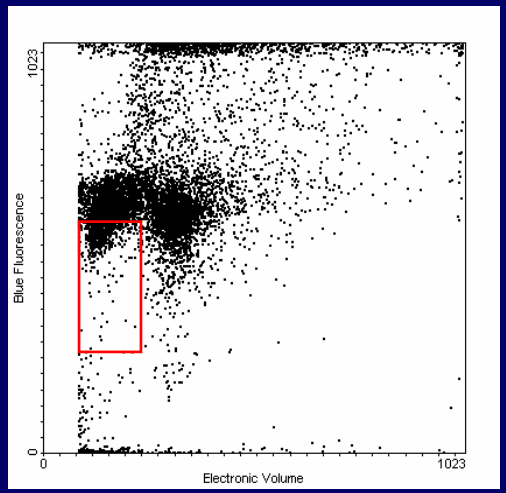
Measurement of **stem cell electronic volume** might provide a valuable new phenotypic marker for stem cell-ness (like scatter does).

side scatter



forward scatter

HO33342 blue

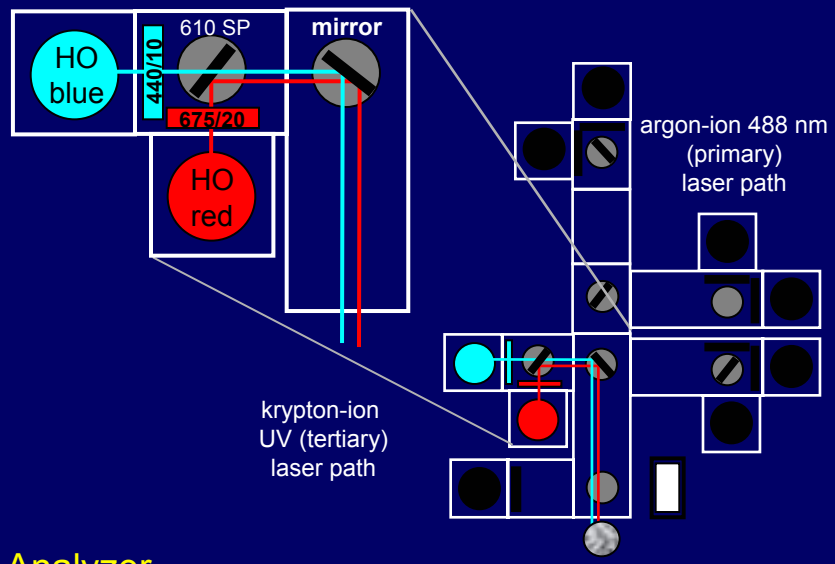


electronic cell volume

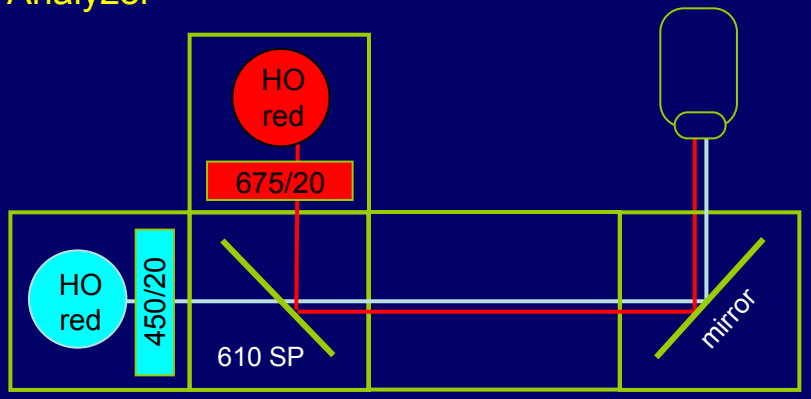
Can we use the NPE Analyzer to measure Hoechst SP?

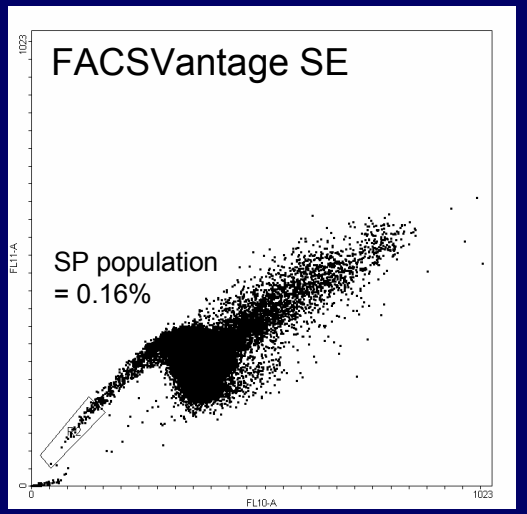
With assistance from Drs. Paul Love and Ella Frolova, NICHD

FACSVantage SE

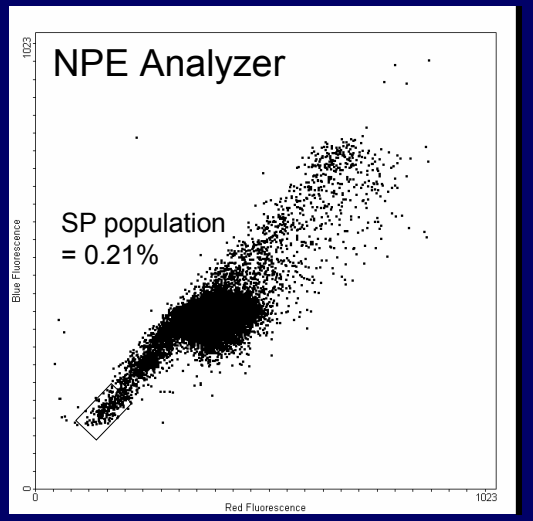


NPE Analyzer





Hoechst 33342 Blue (440/10)



Hoechst 33342 Red (675/20 nm)

Can we use the NPE Analyzer to measure Hoechst SP?

Yes, we can.

But…the same problem as with the violet diode, namely suboptimal excitation. Lower precision … and poorer "junk" separation … than more powerful UV sources.

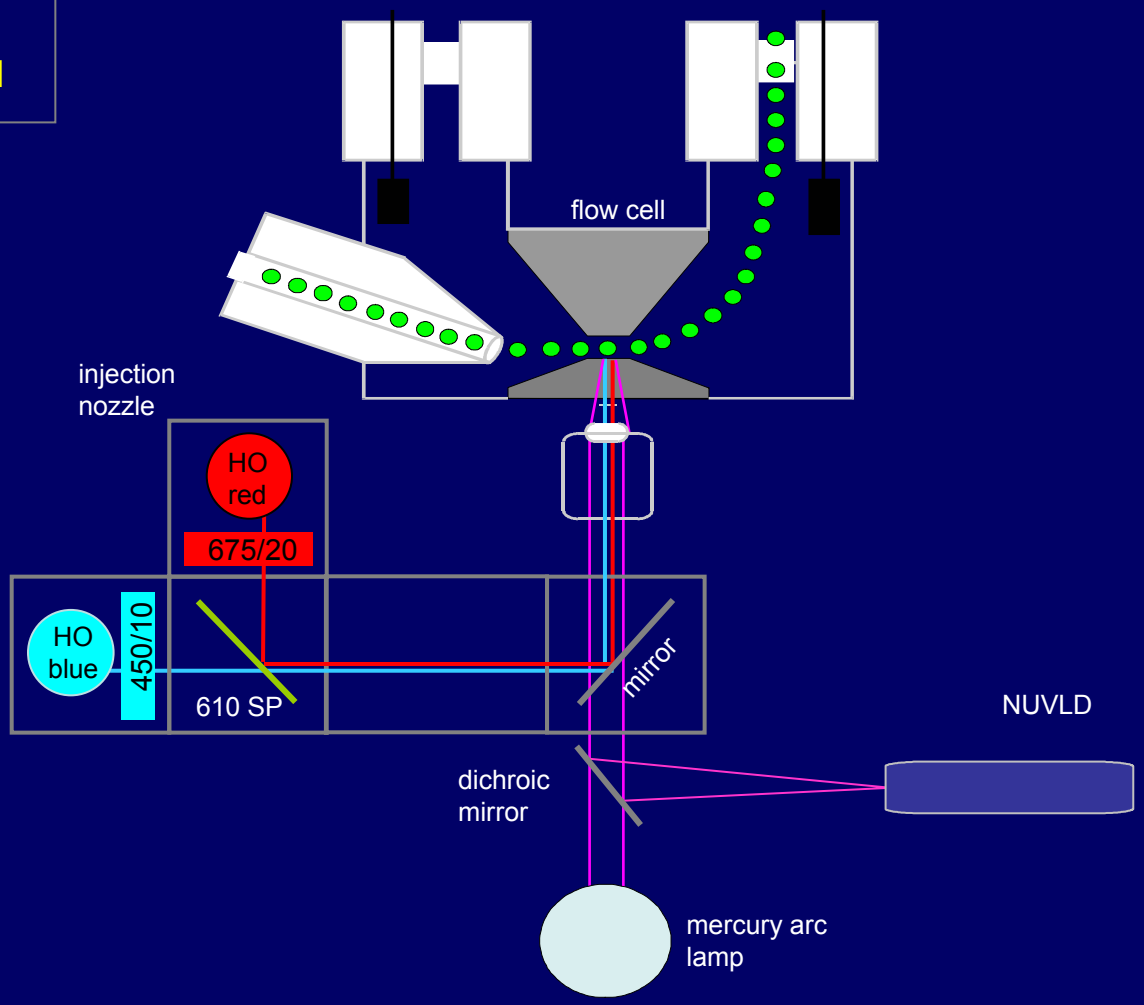
The Hg arc lamp is a thoeretical point source – the actual power level of laser light reaching the flow cell is probably **less than 1 mW**, making it marginal for reproducible SP analysis (we have the same problem with low-power NUVLDs on cuvette flow cytometers)

This is plenty of UV light for DAPI cell cycle…

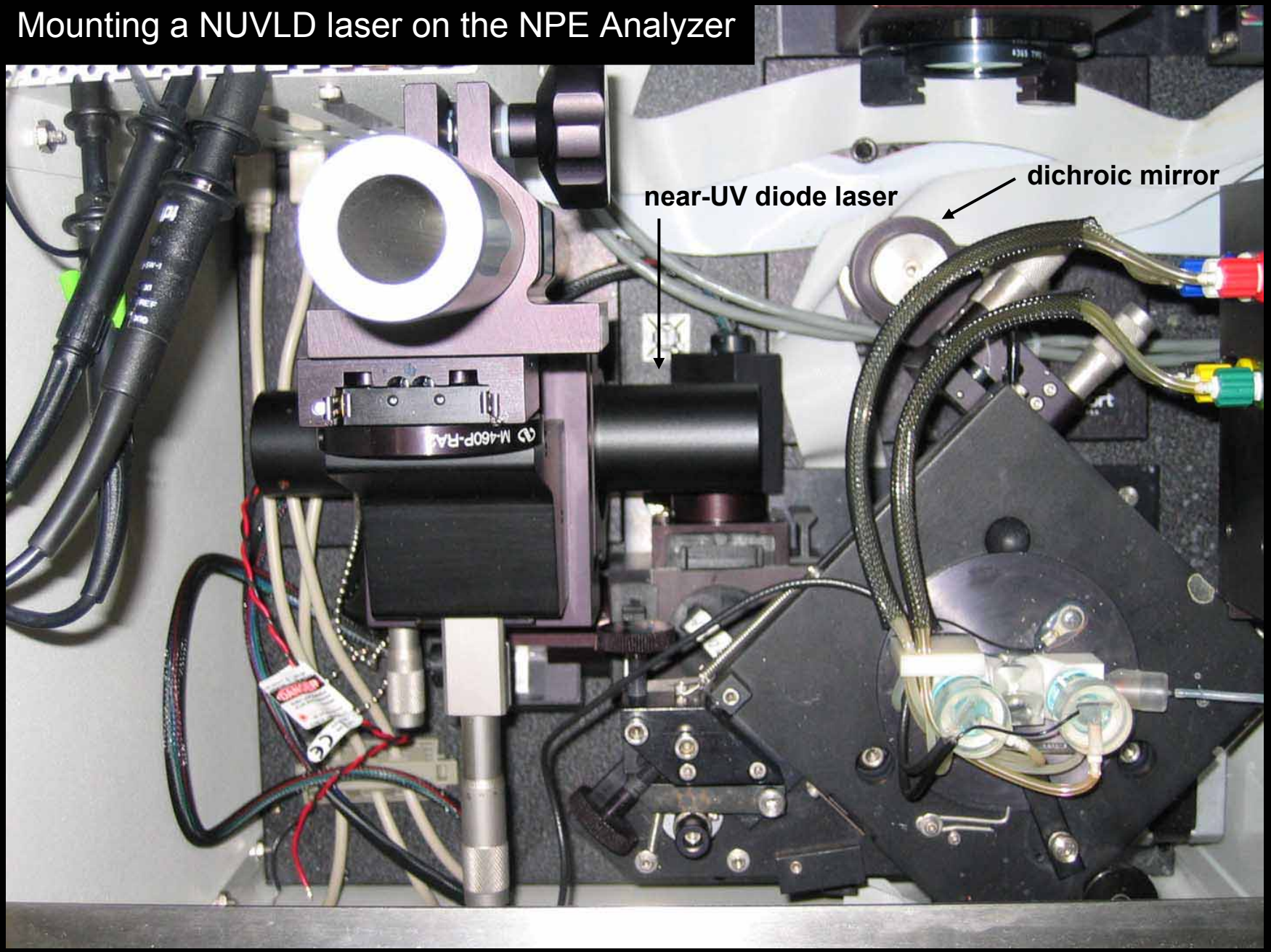
…but less than optimal for applications requiring stronger UV excitation.

Mounting a NUVLD laser on the NPE Analyzer

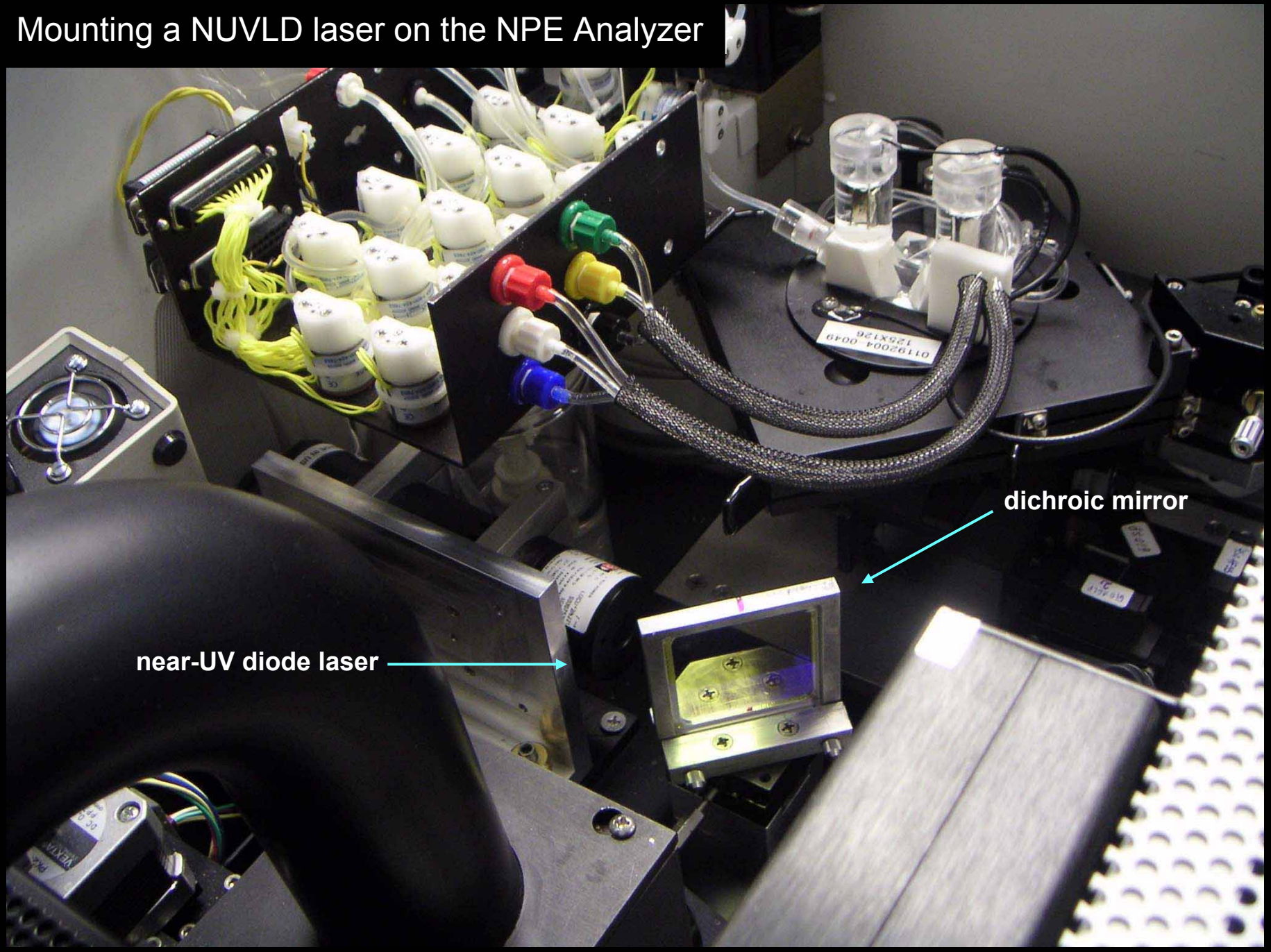
- A NUVLD can be mounted in the NPE Analyzer and the beam steered to the flow cell



Mounting a NUVLD laser on the NPE Analyzer



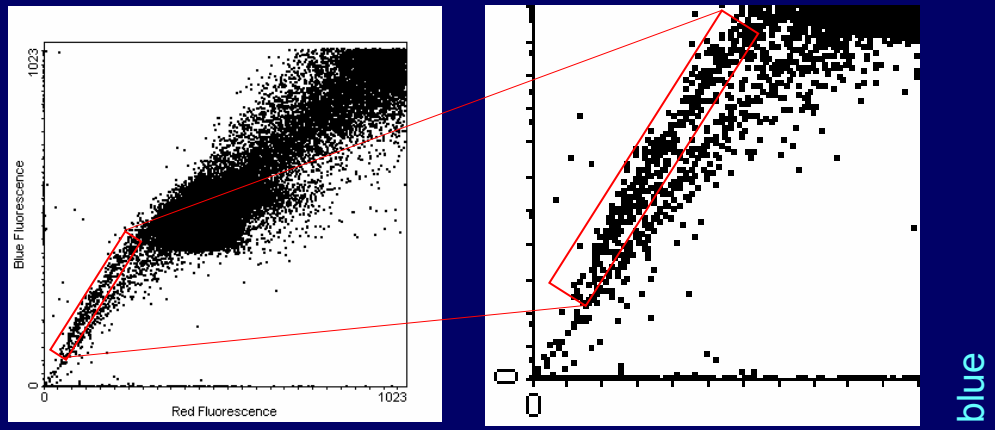
Mounting a NUVLD laser on the NPE Analyzer



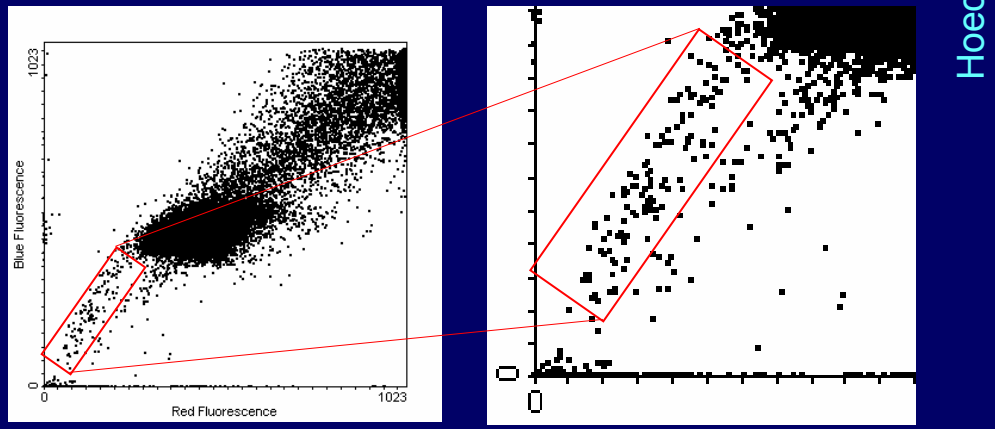
Hoechst SP on the NPE Analyzer

NUVLDs not only give better Hoechst SP resolution, they give greater contrast between the SP population and other non-stem cell hypodiploid populations

NPE Analyzer
Hg arc lamp 365 nm
80 W
HO blue = 450/20
HO red = 675/20



NPE Analyzer
NUVLD 374 nm
7 mW
HO blue = 450/20
HO red = 675/20



Hoechst blue

Hoechst red

Today's Stem Cell Wet Workshop…

We will label whole mouse bone marrow cells and A549 cells (an ABCG2 overexpressing lung carcinoma cell line) with Hoechst 33342 for Hoechst SP analysis.

We will then analyze these cells on the NPE Analyzer, first with the mercury arc lamp UV source, then with the NUVLD.

This is a very new and continually evolving technique … any results we obtain today may have real experimental value.

Acknowledgements

NCI ETIB Flow Lab

Veena Kapoor

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John Vogel
Mark Udey

Molecular Biology Branch, NCI

Michael Bustin
Ella Frolova

Developmental Therapeutics Branch, NCI

Susan Bates
Robert Robey

The University of Miami Medical School

Awtar Krishan

NPE Systems

Richard Thomas
Ernie Thomas
Raquel Cabana
Michael Brochu, Sr.
Michael Brochu, Jr.

Power Technology

James Jackson

BD Biosciences

Larry Duckett
Joe Trotter

Visit our WWW site at…

http://home.ncifcrf.gov/ccr/flowcore/index.htm