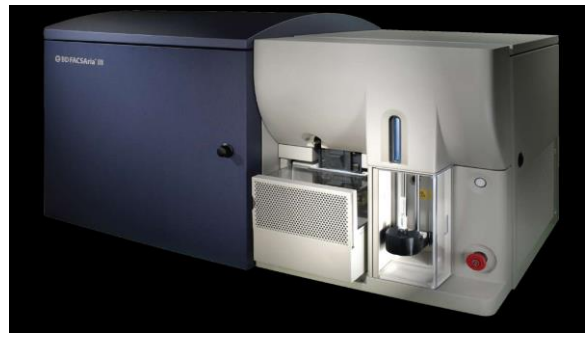
# Principle and Applications of BD FACSAria III



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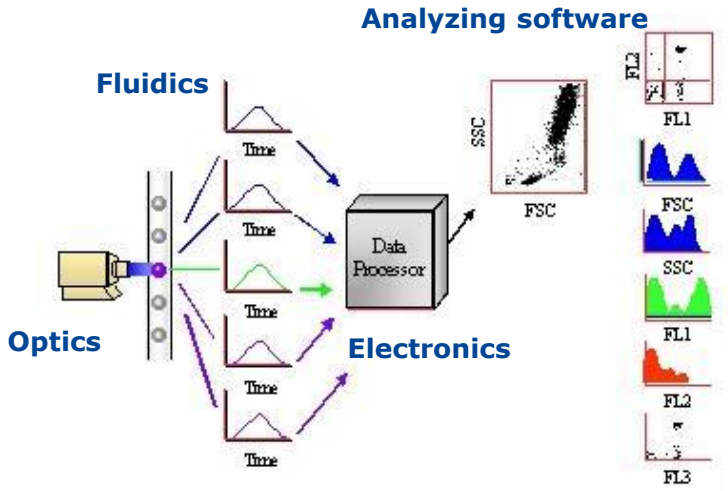
# Principle of Flow Cytometry

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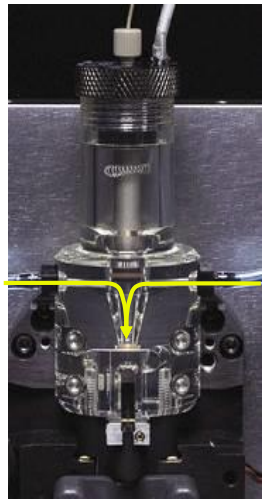
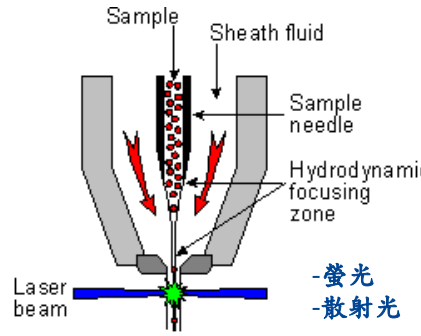
# Flow Cell Structure of FACS Aria III



- Hydrodynamic focus

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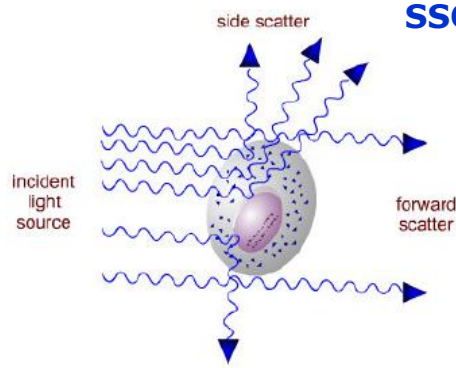
# Scatter Light




**SSC** Internal
Complexity
Granularity

**FSC** Size
Shape
Surface
Refractive index

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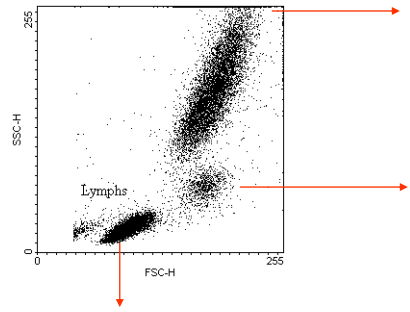
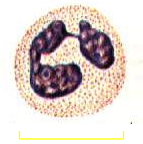
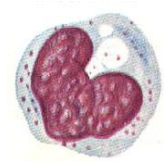
# Scatter Light



**10 to 14 μm**
**Neutrophil**



**15 to 20 μm**
**Monocyte**

**8 to 10 μm**
**Lymphocyte**

**Peripheral blood**

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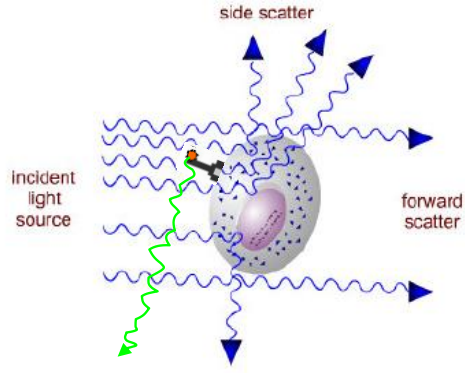
# Fluorescent Light




**Fluorescence**

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# BD FACSAria™ III Filter Guide

| Part # | Dyes | Application | Filters | Mirrors |
|---|---|---|---|---|
| | **488 nm[2]** | | | |
| 343799 | SSC | Cell surface markers, live/dead discrimination, cell cycle | 488/10 | NA |
| 343798 | FITC/Alexa Fluor® 488 | " | 530/30 | 502 LP |
| 343796 | PE | " | 585/42 | 556 LP |
| 344516 | PE-Texas Red®/PI | " | 616/23 | 610 LP |
| 343791 or 343790 | PerCP or PerCP-Cy™5.5 | " | 675/20 or 695/40 | 655 LP |
| 343788 | PE-Cy™7 | " | 780/60 | 735 LP |
| | **561 nm[3]** | | | |
| 649046 | PE/DsRed | Cell surface markers | 582/15 | NA |
| 334934 | PE-Texas Red®, Living Colors®, mCherry, PI | " | 610/20 | 600 LP |
| 334933 or 649422 | PE-Cy™5 or PE-Cy5.5 | " | 670/14 or 710/50 | 630 LP |
| 343788 | PE-Cy7 | " | 780/60 | 735 LP |
| | **633 nm[4]** | | | |
| 343800 | APC/Alexa Fluor® 647 | Cell surface markers | 660/20 | NA |
| 649618 | Alexa Fluor® 700 | " | 730/45 | 690 LP |
| 343788 | APC-Cy7 or APC-H7 | " | 780/60 | 735 LP[5] |

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# BD FACSAria™ III Filter Guide

| Part # | Dyes | Application | Filters | Mirrors |
|---|---|---|---|---|
| | **375 nm** | | | |
| 642329 | DAPI/Hoechst Blue | Cell cycle | 450/20 | NA |
| 642328 | Hoechst Red | Side population | 670 LP | 610 LP |
| | **405 nm** | | | |
| 343801 | BD Horizon™ V450, Pacific Blue™, DAPI | Cell surface markers | 450/40 | NA |
| 649421 | BD Horizon™ V500, AmCyan | " | 510/50 | 502 LP |
| | Qdot[1] 565 | " | 560/40 | 545 LP |
| | Qdot 585 | " | 585/42 | 555 LP |
| | Qdot 605 | " | 610/20 | 600 LP |
| | Qdot 655 | " | 670/30 | 635 LP |
| | Qdot 700 | " | 710/50 | 685 LP |
| | Qdot 705 | " | 710/50 | 685 LP |
| | Qdot 800 | " | 800/30 | 735 LP |
| | **445 nm** | | | |
| 648921 | SSC | Cell surface markers, live/dead discrimination, cell cycle | 445/15 | NA |
| 648922 | CFP, AmCyan fluorescent protein | " | 510/80 | 470 LP |

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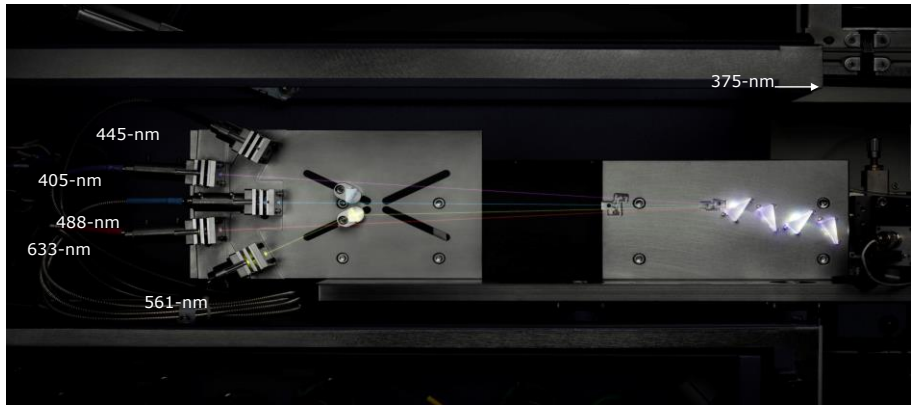
# Optical Plate

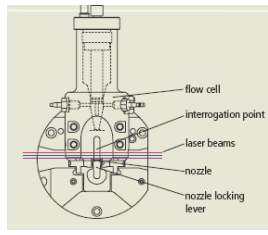



# Aria III Optics Detector

**405-nm or 375-nm**
- up to 8 colors

**633-nm**
- up to 3 colors

**488-nm or 445-nm**
- two colors w / 561-nm
- up to six colors w/o 561-nm

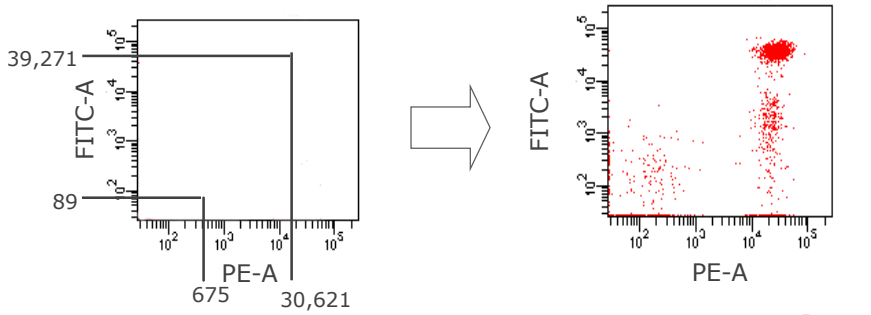



# Data Storage

List-Mode Data

| | Time | FSC | SSC | FITC | PE |
|---|---|---|---|---|---|
| Event 1 | 0 | 60 | 120 | 89 | 675 |
| Event 2 | 10 | 160 | 65 | 39,271 | 30,621 |
| Event 3 | 30 | 650 | 160 | 22,688 | 6,189 |

May be exported as FCS file

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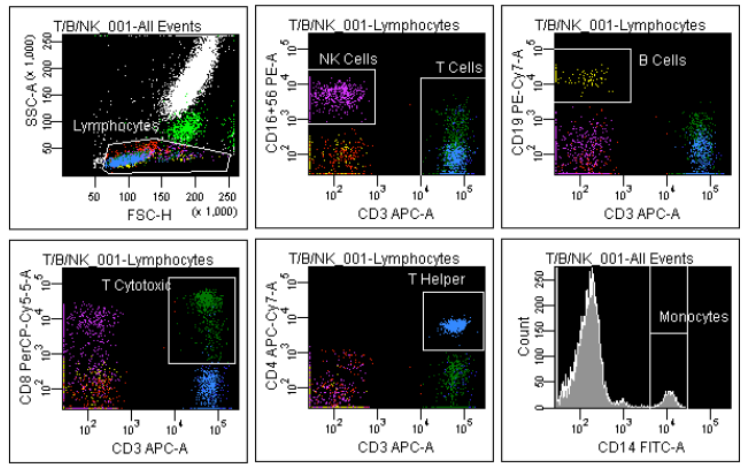
# Multi-parameter Immune Cell

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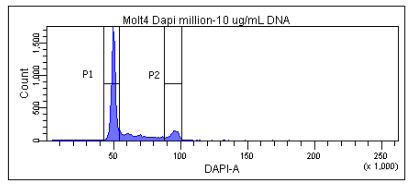
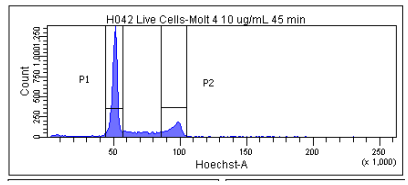
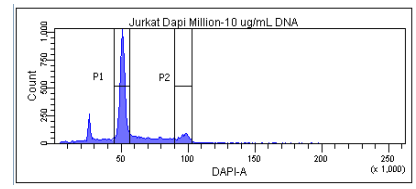
# Cell Cycle Analysis

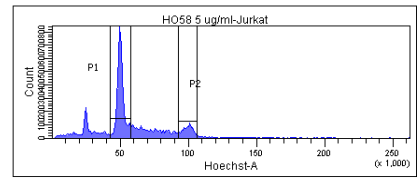
Molt 4




Jurkat

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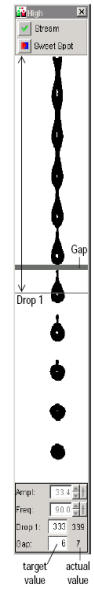
# Outline

- Flow cytometery principle
- Flow Sorting principle
- Flow Sorting as a research tool
- Expected Output

BD

# Drop Formation

- Amplitude
  - intensity of the drop drive
- Frequency
  - number of drops formed per second
- Drop 1
  - the first disconnected drop
- Gap
  - Number of pixels between the stream breakoff and the first drop

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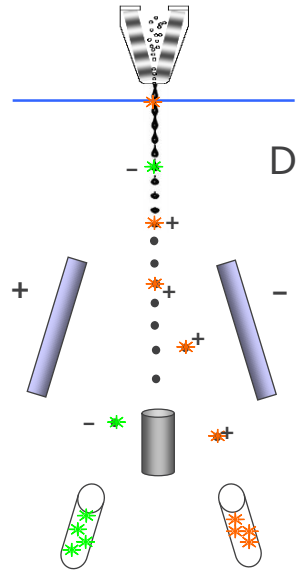
# Drop Charging

Drop Charging

Drop generation



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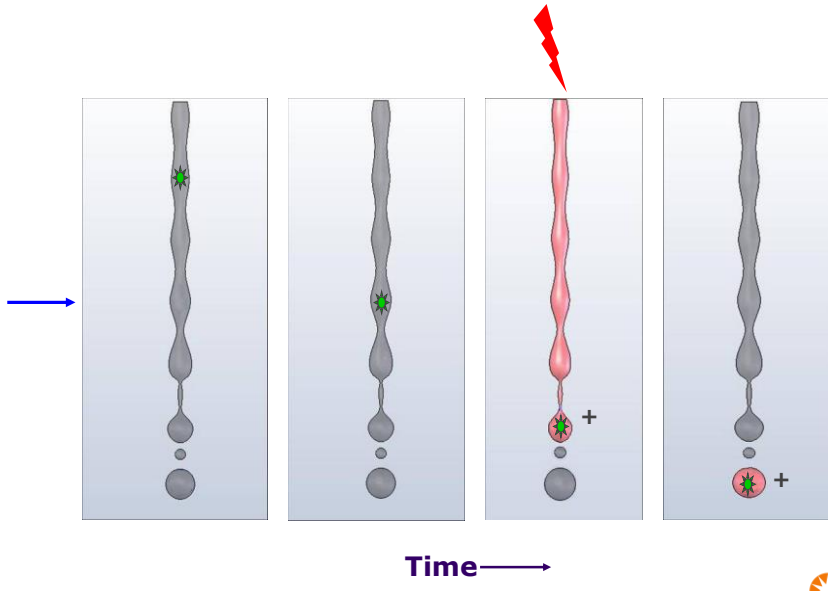
# Drop Charging Delay

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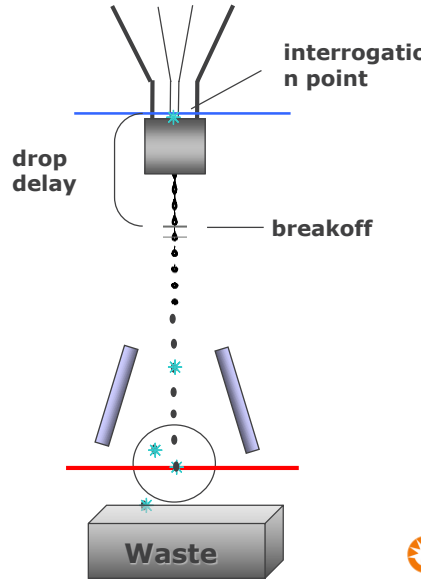
# Drop Delay

BD FACS™ Accudrop technology

- Accudrop beads
- Diode laser
- Camera
- Optical filter

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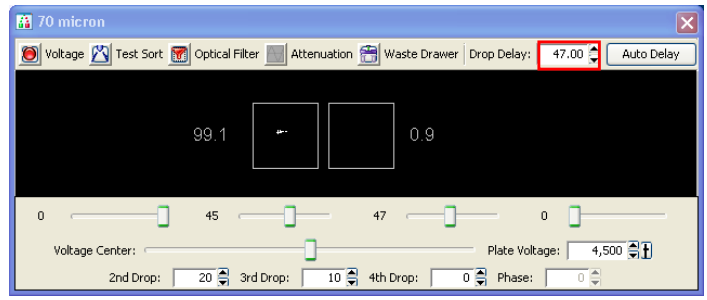
# Setting the Drop Delay

Adjust drop delay until ~100% are sorted to the left.




# Overall Sort Setup Review

1. Sample generates light scatter and fluorescent signals; signals are analyzed.

2. Charge is applied via the stream-charging wire.

3. Charged droplets break off.

4. Deflection plates attract or repel charged droplet.

5. Uncharged droplets pass to waste.

6. Charged drops containing particles of interest are collected.

## Outline

- Flow cytometery principle
- Flow Sorting principle
- Flow Sorting as a research tool
- Expected Output

BD

## Gating Target cell

- Life or death
- Morphology
- Surface antigens
- Gene expression.
- Cell functions.
- DNA content.
- Specific DNA sequence.

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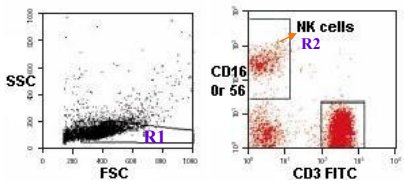
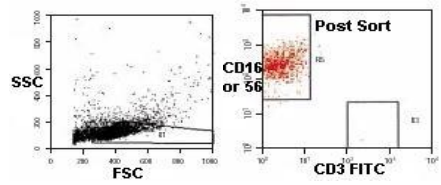
# Sorting Cells By Surface Markers

- **Sorting NK Cells**
  - CD3 FITC to exclude T cells
  - CD56+CD16 PE to include all NK Cells.

分選前的分析




分選後結果

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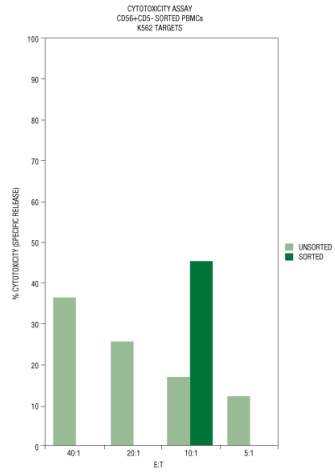
# Sorting NK Cells for Cytotoxicitity Studies

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# Regulatory T Cell Sorting

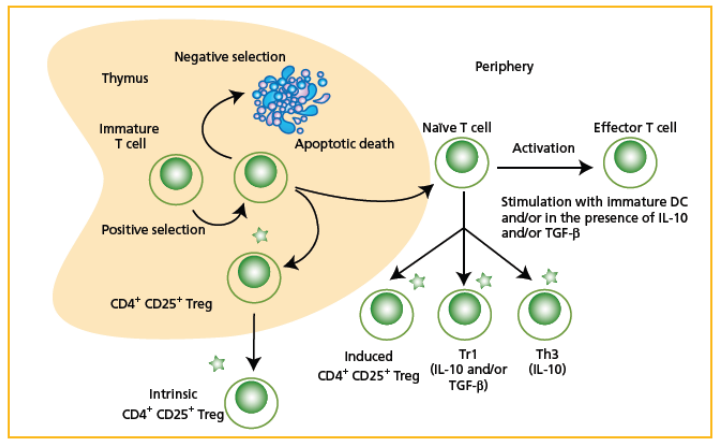


Figure 1. Schematic representation of the role of regulatory T cells in immune function

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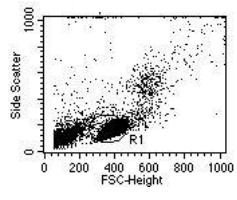
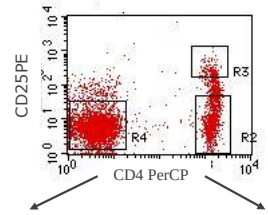
# CD4/CD25 Treg sorting



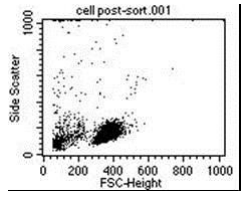



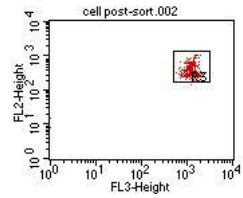

1.35 %

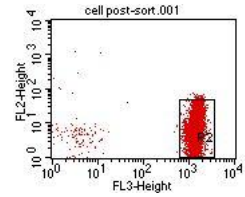
8.94 %

Sort on R1 and R3

sort on R1 and R2

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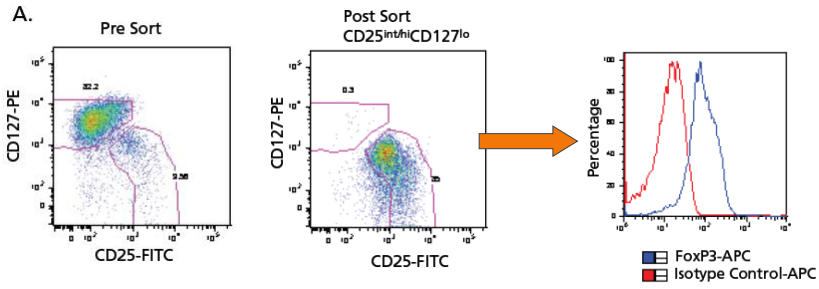
# CD4/CD25 High/CD127 Lo

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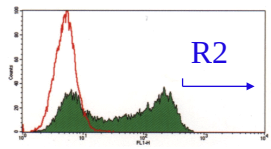
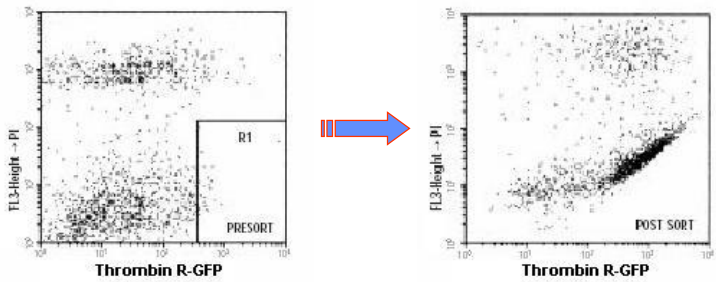
# Sorting by Gene Expression

**Add viability check.**

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# Rare Cell Sorting tip

- Rare cell sorting usually requires pre-enrichment steps:
  - Bring the starting purity to > 5 %
  - Ficoll
  - Immune Panning
  - Magnetic Beads (Positive/Negative)

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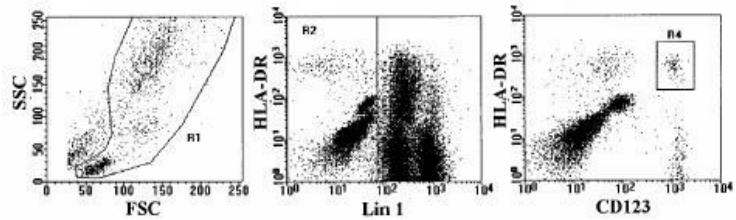
# Rare Cell Sorting

| Parameter | CD123+ DCs | CD11c+ DCs | Basophils |
|---|---|---|---|
| Lin 1 | - | -/+ | - |
| Anti-HLA-DR | ++ | +++ | - |
| CD123 | +++ | + | +++ |
| CD11c | - | +++ | + |

Immunophenotype of CD11c, CD123 DCs and baso (fluorescence intensities)

- **4-Color Staining**
  - Lineage Cocktail FITC
    - CD3, 14, 16, 19, 20, 56
  - CD123 (IL-3Rα) PE
  - HLA-DR PerCP
  - CD11c APC
  - Controls
  - FACLysing Solution

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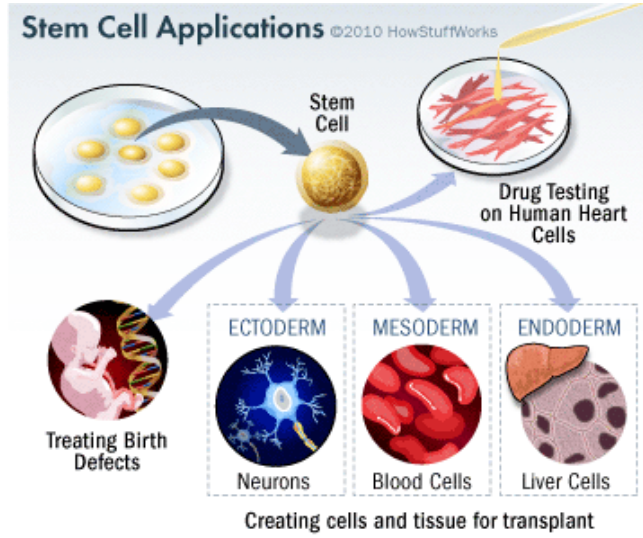
# Stem cell sorting




Schematic adapted from http://stemcells.nih.gov/index.asp

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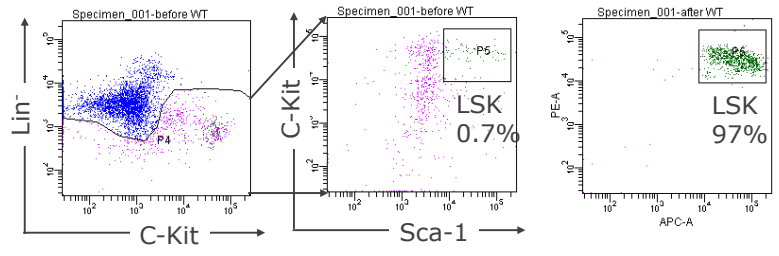
# Three Color-staining for Defining and Sorting Mouse LSK Bone Marrow Stem Cell

**Before Sorting** **After Sorting**

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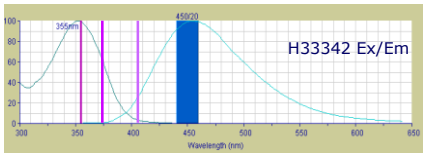
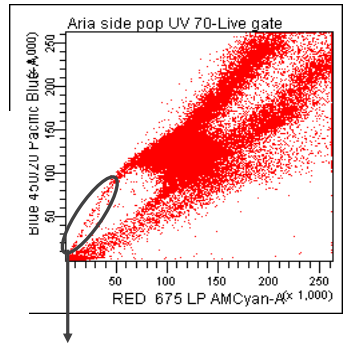
# Alternative: Isolation of Mouse HSC using Side Population Staining

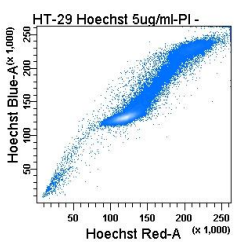
- The Side Population (SP) assay is a method that makes use of the ability of primitive progenitor cells to efflux the vital DNA dye Hoechst 33342
  - *Described by Goodell and co-workers*
  - Multi-drug-like transport mechanism
- H33342 is excited by a UV laser and fluorescence measured at two wavelengths
  - Red fluorescence (>630 nm, >610 nm…)
  - Blue fluorescence (450/20 or 450/40 BP)

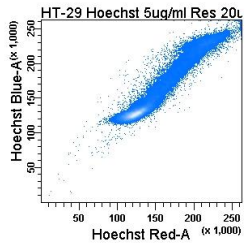


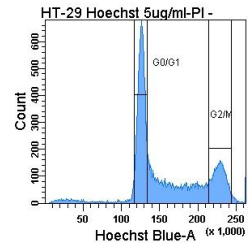




Collected Stem Cells can be cultured and used for further experiments

# Cancer Cell Line Side Population



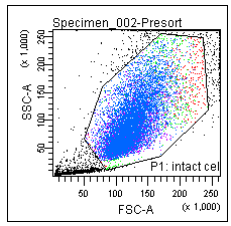



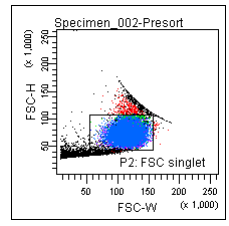



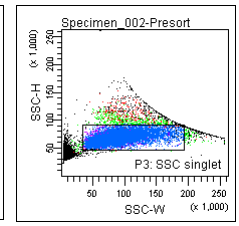

**Cancer Cell Line Side Population**
The human HT-29 colon cancer cells were stained with Hoechst 33342 and run on the FACSAria III equipped with a Near UV 375nm laser.
An ABC transporter blocker was used as a control

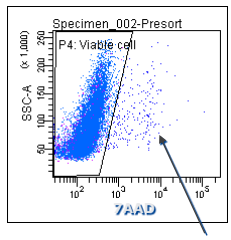
# EPC Cell sorting and tube formation

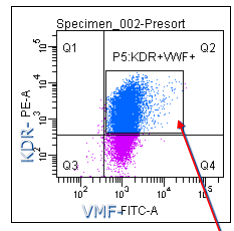
- Pre-sort analysis
- Set gating hierarchy and check target cell purity




Tube: Presort

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 15,112 | #### | 100.0 |
| P1: intact cell | 10,964 | 72.6 | 72.6 |
| P2: FSC singlet | 10,679 | 97.4 | 70.7 |
| P3: SSC singlet | 10,239 | 95.9 | 67.8 |
| P4: Viable cell | 9,811 | 95.8 | 64.9 |
| Q1 | 25 | 0.3 | 0.2 |
| Q2 | 7,339 | 74.8 | 48.6 |
| Q3 | 46 | 0.5 | 0.3 |
| Q4 | 2,401 | 24.5 | 15.9 |
| P5:KDR+VWF+ | 7,052 | 71.9 | 46.7 |

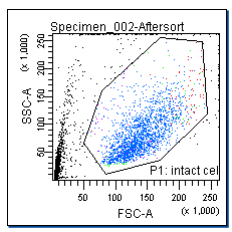
Dead cell

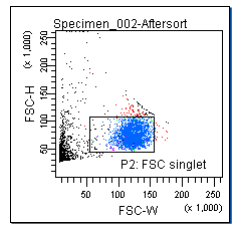
Target cell

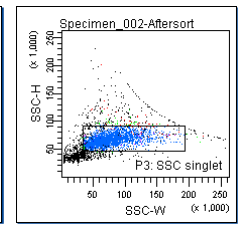
BD

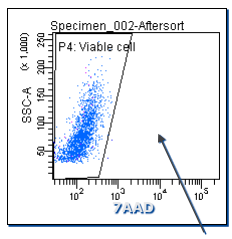
# EPC Cell Line sorting and tube formation

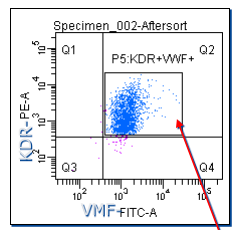
## After sort




Tube: Aftersort

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 3,422 | #### | 100.0 |
| P1: intact cell | 2,162 | 63.2 | 63.2 |
| P2: FSC singlet | 2,096 | 96.9 | 61.3 |
| P3: SSC singlet | 2,045 | 97.6 | 59.8 |
| P4: Viable cell | 1,998 | 97.7 | 58.4 |
| Q1 | 3 | 0.2 | 0.1 |
| Q2 | 1,956 | 97.9 | 57.2 |
| Q3 | 4 | 0.2 | 0.1 |
| Q4 | 35 | 1.8 | 1.0 |
| P5:KDR+VWF+ | 1,938 | 97.0 | 56.6 |

Dead cell

Target cell

BD

# Outline

- Flow cytometery principle
- Flow Sorting principle
- Flow Sorting as a research tool
- Expected Output

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# 期望分選結果

- 分選細胞純度高達 **95%** 以上
- 分選細胞應仍保持存活度
- 分選細胞可保無菌
- **Cell function may not be changed by staining.**
- **Cell viability is not affected by sorting process.**

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# 分選結果之評估

分選10,000顆細胞，其細胞原始純度為10%
-理想的分選結果為1000顆細
-收集管內共計有960顆細胞
-分選後分析符合分選條件的細胞共計950顆
-儀器顯示分選細胞總數為975顆

- 純度(Purity)：
  符合分選條件的物件數目/物件總數　950/960 = 99%
- 回收率(Recovery)：
  實際分選標的細胞總數/儀器計算總數　950/975 = 97%
- 產率(Yield)：
  實際分選標的細胞總數/理想分選總數　950/1000 = 95%

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# 影響分選結果的因素

- Particle rate
- Drop drive frequency
- Frequency of the target population
- Sort mode

  Purity：High Purity and Recovery
  Enrich: For Enrichment

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# 樣品製備範例

- 所需準備之細胞量計算:
  - 若原始純度15%
  - 樣品濃度：$1X10^7$/ml
  - 低速分析速度：2000/s
  - 分選速度：300/s。
  - 每30 min可得約$5.4x10^5$細胞。
- 原始純度降低應調高樣品濃度。
- 原始純度低於5%則需先濃集處理。

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# 樣品製備注意事項

- 高壓會使buffer的pH值下降, 進而影響細胞的存活. 所以建議於phenol red -free sample buffer中添加25mM HEPES可使環境的pH值維持一定值.
- sample buffer中的protein含量也會影響分選純度. 若是建議使用5% FCS, 則可改用0.5% - 2% BSA取代以獲得較好結果.
- Collection tube內置放適合細胞存活的cold culture media with higher concentration of FCS.
- Collection tube管壁請先coating 1 - 4% BSA overnight.
- 分選前，樣品先加入viable dye (例如 7-AAD)，以避免分選已死亡細胞。分選後，也應取出一些細胞加入7-AAD再上機確認細胞存活狀況，以改善分選過程中是否有任何疏失之處。
- 樣品必須經35 μm濾網以避免塞管

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# 樣品製備注意事項

- 儘量多次低速離心以去除細胞碎片與小雜物
- 細胞直徑之考量
- 分選時間不應太長, 即分多管收集細胞. 建議測試不同的分選時間長短(10min, 20min, 或30min)以了解是否會影響細胞的活性與隨後的培養結果.
- Side stream應避免打在管壁上.
- 應使用NA/LE(no azide, low endotoxin) reagent標識細胞. 若是無法找到此類試劑, 則細胞分選下來後應離心清洗多次以去除NA及endotoxin. 離心條件:4°C, 1000rpm.
- 單一clone細胞之培養較mixed population不易, 主要是適合細胞生長所需環境與營養因子太複雜. 以傳統培養hybridoma clones來說, 通常是需要加入feeder cells或是conditioned media. 可試著使用conditioned media加入culture media(5%, 10%或20%)中來加強培養環境.

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