# Flow cytometry tools for characterization of GMP cell products

*Alexandra Rizzitelli, PhD*

*Project manager*

*Cell therapies Pty Ltd, Melbourne, Australia*

# What is product characterization?

- Identity (phenotype)
  - Surface markers
  - Gene expression
- Potency (efficacy)
  - Cytokine release
  - Killing assay
- Purity (contaminants) & impurity
  - Endotoxins
  - BSA
  - FCS
  - Other cells
- Safety (sterility)
  - Mycoplasma
  - Bacteria
  - Virus
  - Fungi

→ Ensure product quality, specification, lot-to-lot consistency
→ Complexity & heterogeneity

# What is GMP?

- Defined manufacturing process

- Good Manufacturing Practices aims:
  - quality in each batch of product during all stages of the manufacturing process
  - TGA (Therapeutic Goods Administration) standards = control over the process
    - Donor selection / screening/ collection
    - Materials, equipment and reagents used
    - Transport, storage and shelf life
    - Labelling
    - Tests performed: FIO # release

# Flow in GMP environment

- Identity
  - Multi-parametric  cell surface /intracell staining (B, T cells, etc….)
  - Gene expression (mRNA, miRNA, etc…)
  - Viability
  - Count
- Impurity
- Potency
  - Intra-cellular cytokine production
  - Killing assay
- Affordable / Quick / Accessible  -> primary choice

# Flow in GMP environment

- Multiple platform
  - Instrument?
  - Analyser or sorter? -> open system
- Reagents grade
  - In Vitro Diagnostic (IVD)
  - Research use only (RUO)
    - Level of testing / specification (e.g Ab concentration)
- Flow in GMP = control of facility + instrument + material + reagents + method

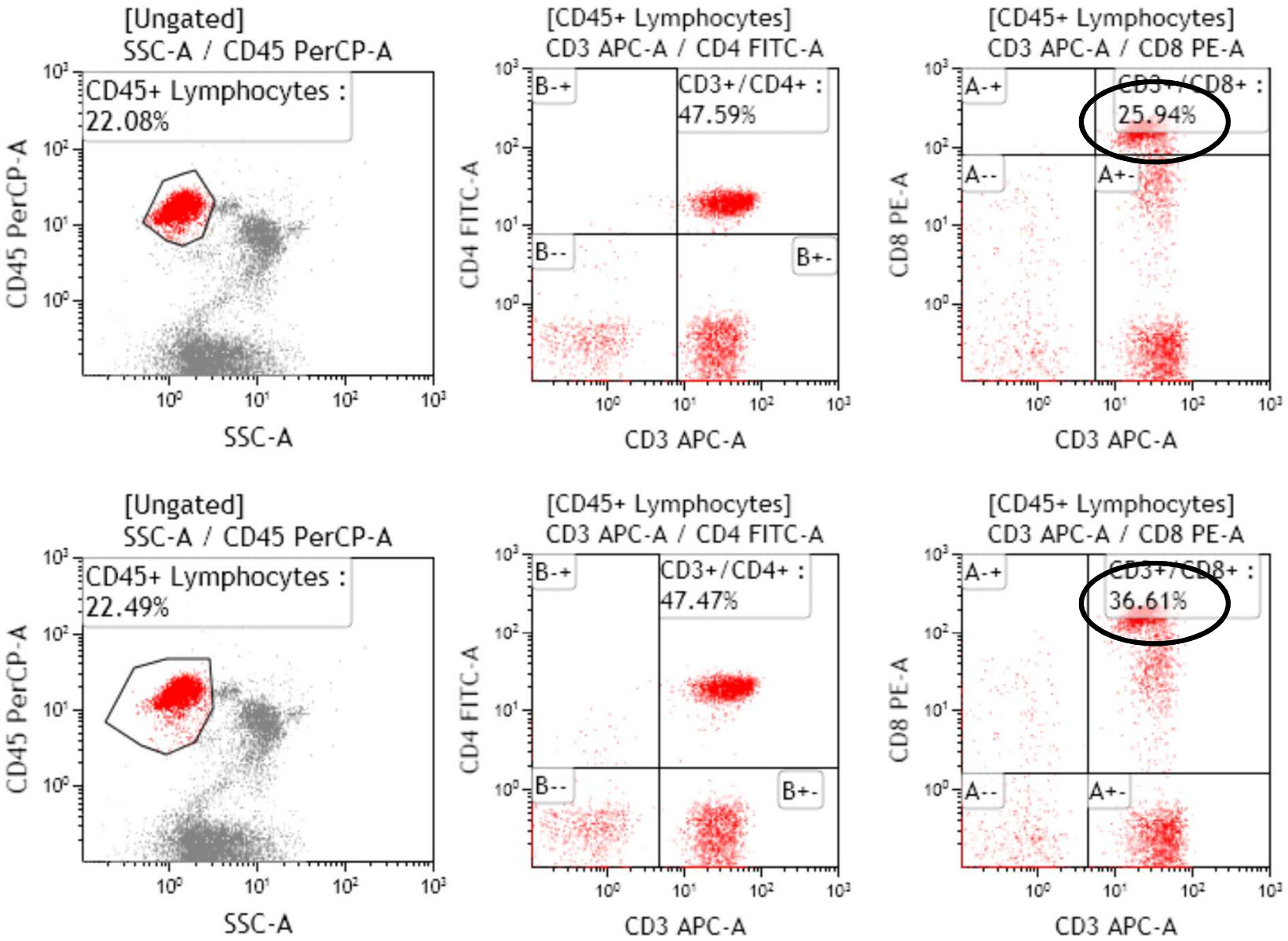
# Flow in GMP environment

- Evidences of:
  - Staff training
  - Procedure followed
  - Records
  - Control of material
  - Defined analysis steps
- Facility / instrument:
  - Temperature
  - Humidity
  - Cleaning
  - Maintenance
  - Performance (daily CST)

# Validation of flow methods

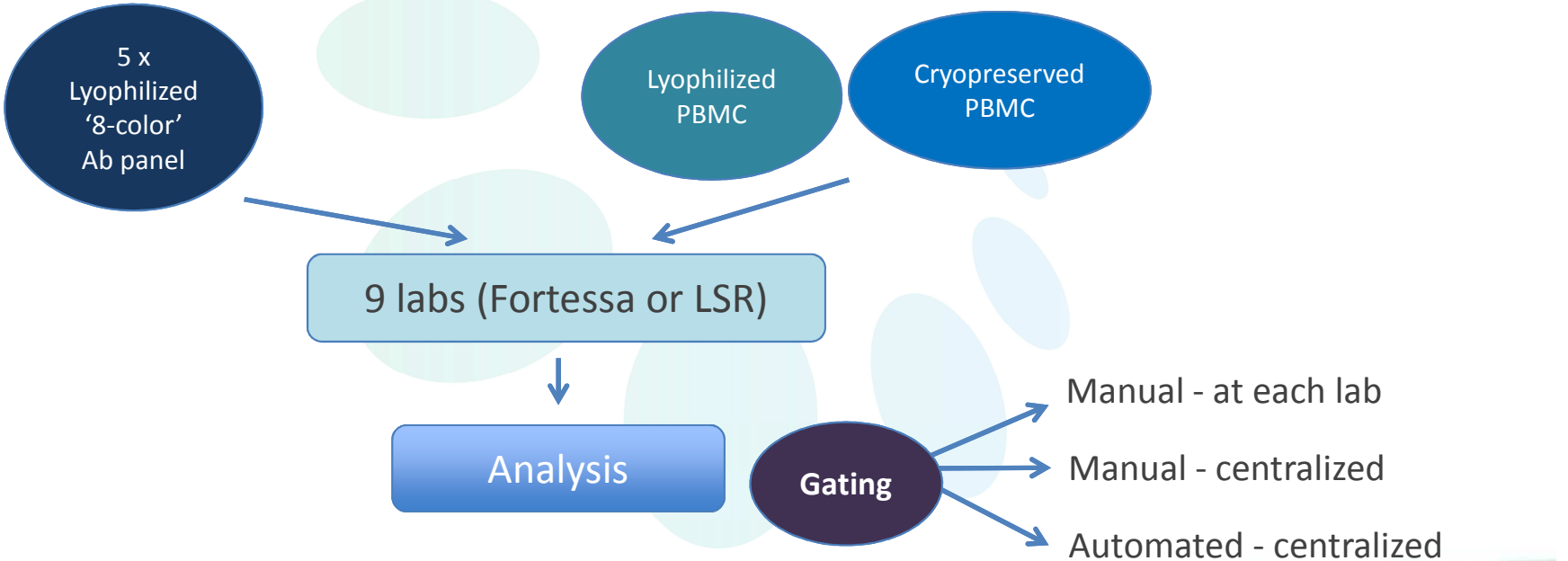
- Validation is “to demonstrate that the analytical method is suitable for its intended purpose” *ICH Guideline, validation of analytical procedures: text and methodology q2(r1) 2005*
- Validation characteristics:
  - Specificity -> matrix interference
  - Accuracy -> agreement with a conventional value -> NO universal reference for flow
  - Precision
    - repeatability :short time interval, intra-assay
    - intermediate precision: within laboratory, different days, operators, instrument
    - reproducibility : between laboratories
  - Range -> upper, lower concentration
  - Linearity -> within a range
  - Detection Limits
  - Quantification Limits
  - Robustness -> unaffected by small, deliberate variations (time)
- Other to consider:
  - Multisite standardisation
  - Control/reference sample
  - Sample preparation
  - Analysis, gating strategy

# Subjective analysis - Gating



# Standardizing gating

- Finak et al. Nature scientific report Feb 2016
  - Human Immune Phenotyping Consortium (HIPC)




**OpenCyto and FlowDensity**

# Standardizing gating

- Instruments standardized:
  - Target values for PMTV
  - Compensation beads lyophilized

- Findings
  - Lyophilized cells slightly better than cryopreserved
  - Manual centralized more reproducible than per-site manual gating
  - Automated gating is as good as manual centralized gating - except for not well defined population (dim/rare)

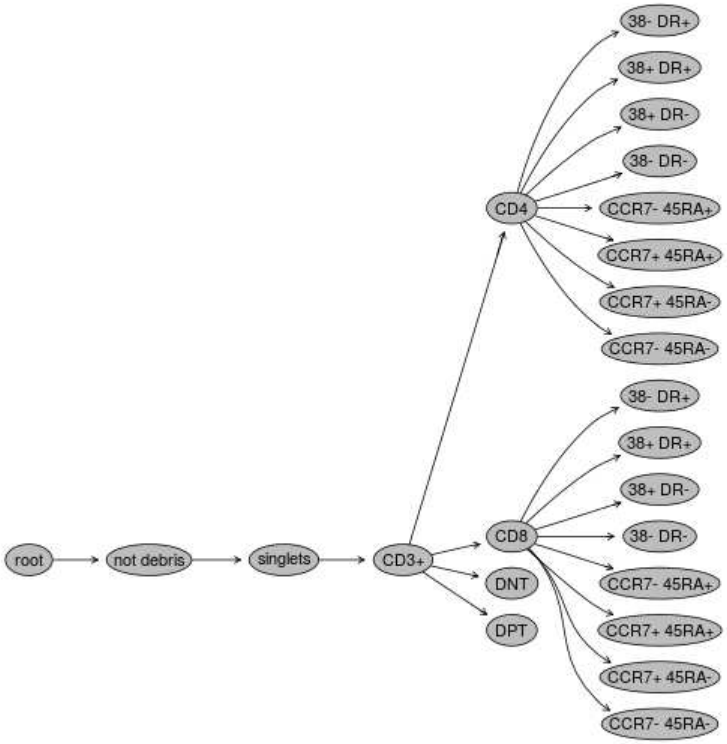
# OpenCyto / FlowDensity

- Open Source packages using R language
- Series of written commands define gating strategy based on algorithm models
- 1 Ab panel -> 1 strategy -> 1 file saved
- Applicable to multiple experiment as long as same markers and fluorochromes are used in the sample

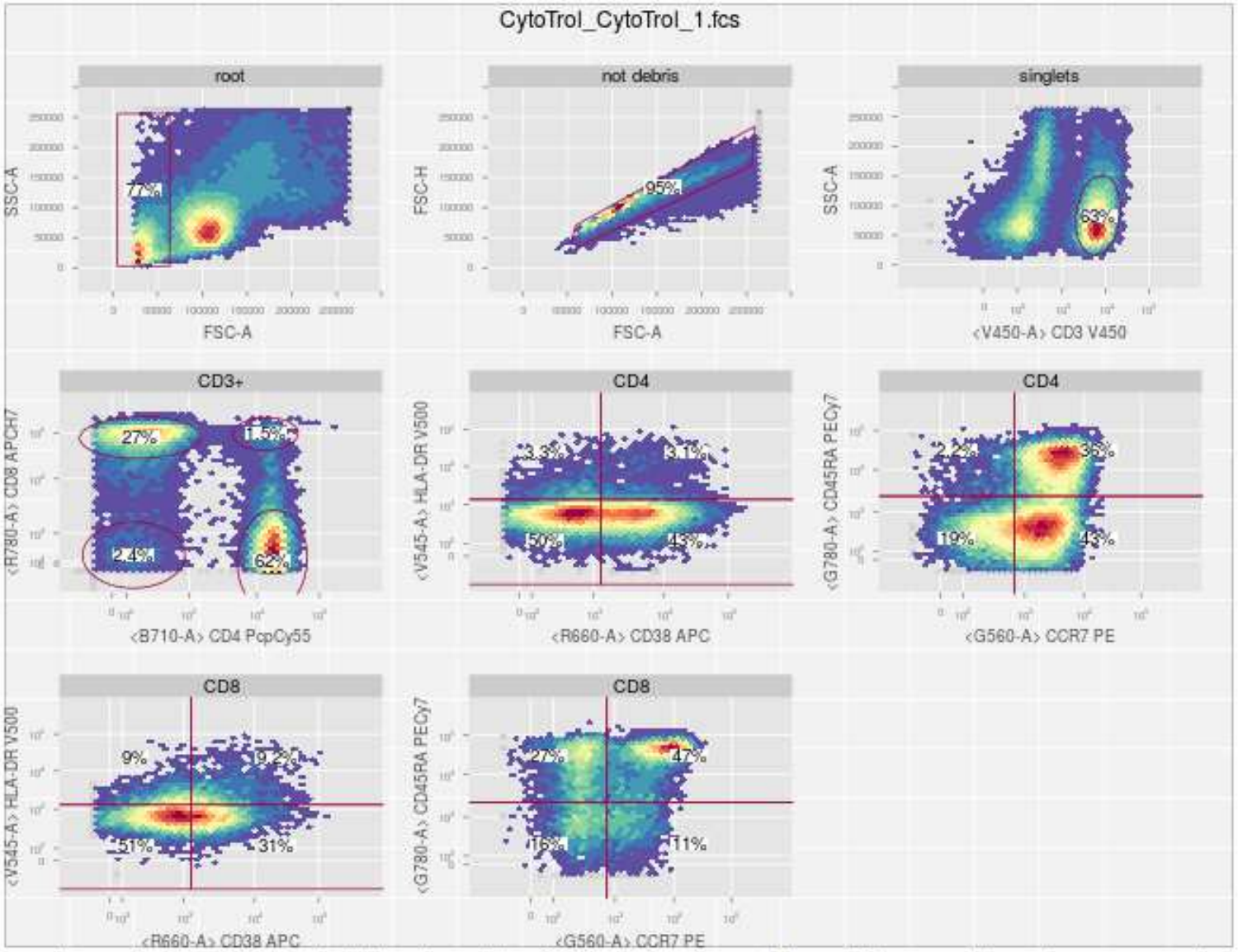
# OpenCyto

```
##             alias              pop    parent        dims gating_method
##  1:     nonDebris        nonDebris      root       FSC-A    mindensity
##  2:      singlets         singlets nonDebris FSC-A,FSC-H   singletGate
##  3:         lymph            lymph  singlets FSC-A,SSC-A     flowClust
##  4:           cd3              cd3     lymph         CD3    mindensity
##  5:             *     cd4-/+cd8+/-       cd3     cd4,cd8    mindensity
##  6: activated cd4        CD38+HLA+  cd4+cd8-    CD38,HLA      tailgate
##  7: activated cd8        CD38+HLA+  cd4-cd8+    CD38,HLA      tailgate
##  8:      CD45_neg           CD45RA-  cd4+cd8-      CD45RA    mindensity
##  9:     CCR7_gate             CCR7+  CD45_neg        CCR7     flowClust
## 10:             * CCR7+/-CD45RA+/-  cd4+cd8- CCR7,CD45RA       refGate
## 11:             * CCR7+/-CD45RA+/-  cd4-cd8+ CCR7,CD45RA    mindensity
##              gating_args collapseDataForGating groupBy
##  1:                                         NA       NA
##  2:                                         NA
##  3: K=2,target=c(1e5,5e4)                   NA
##  4:                                       TRUE
##  5:     gate_range=c(1,3)                   NA
##  6:                                         NA
##  7:              tol=0.08                   NA
##  8:     gate_range=c(2,3)                   NA
##  9:           neg=1,pos=1                   NA
## 10:    CD45_neg:CCR7_gate                   NA
## 11:                                         NA
##     preprocessing_method preprocessing_args
##  1:                                     NA
##  2:                                     NA
##  3:      prior_flowClust                NA
##  4:                                     NA
##  5:                                     NA
##  6:  standardize_flowset                NA
##  7:  standardize_flowset                NA
##  8:                                     NA
##  9:                                     NA
## 10:                                     NA
## 11:                                     NA
```

```
gh <- gs[[1]]
plot(gh)
```



# OpenCyto

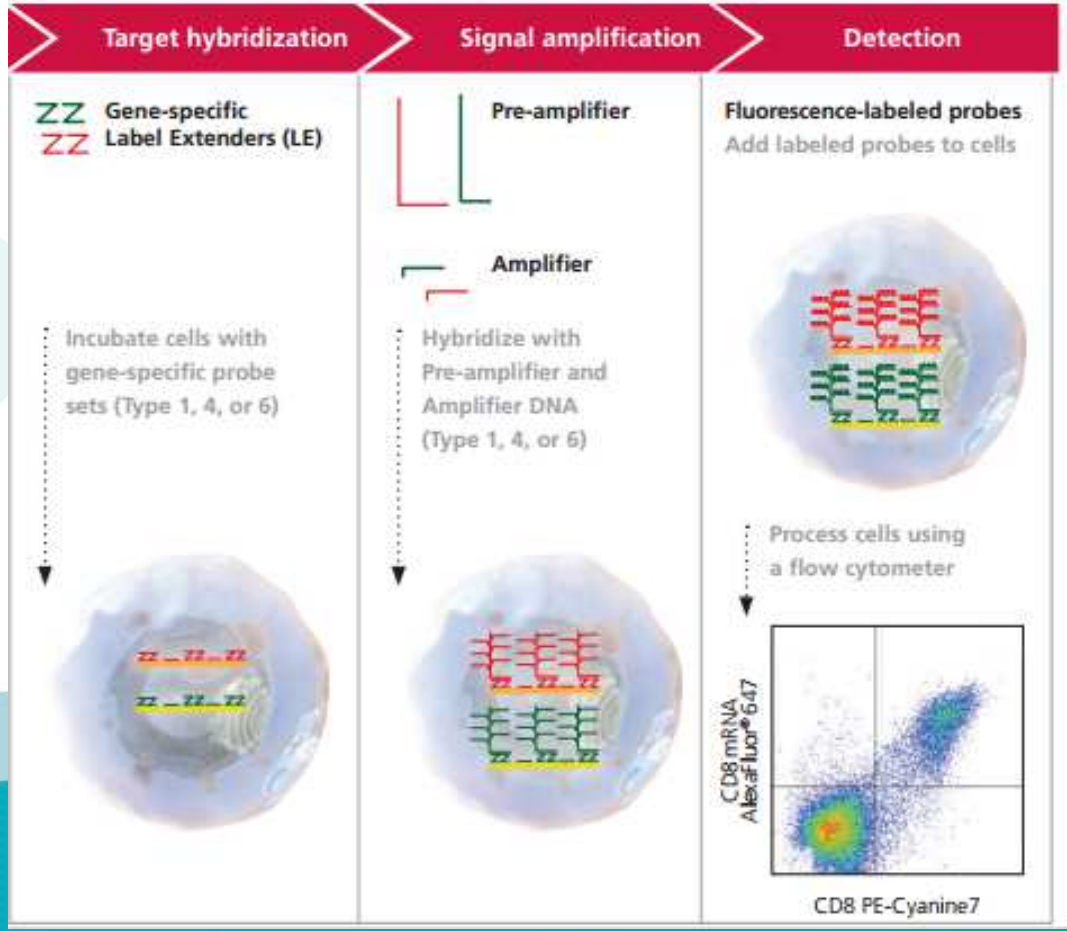

CytoTrol_CytoTrol_1.fcs
root
SSC-A
FSC-A
77%
not debris
FSC-H
FSC-A
95%
singlets
SSC-A
‹V450-A› CD3 V450
63%
CD3+
‹R780-A› CD8 APCH7
‹B710-A› CD4 PcpCy55
27%
2.4%
62%
CD4
‹V545-A› HLA-DR V500
‹R660-A› CD38 APC
3.3%
50%
43%
CD4
‹G780-A› CD45RA PECy7
‹G560-A› CCR7 PE
2.2%
19%
43%
CD8
‹V545-A› HLA-DR V500
‹R660-A› CD38 APC
9%
51%
31%
CD8
‹G780-A› CD45RA PECy7
‹G560-A› CCR7 PE
27%
47%
16%
11%

# Automatic gating and GMP

- Strength:
  - No subjectivity
  - Increase reproducibility
  - Audit
  - Time / cost for large dataset
- Validation:
  - Software *(FDA, 21 CFR part 11)*

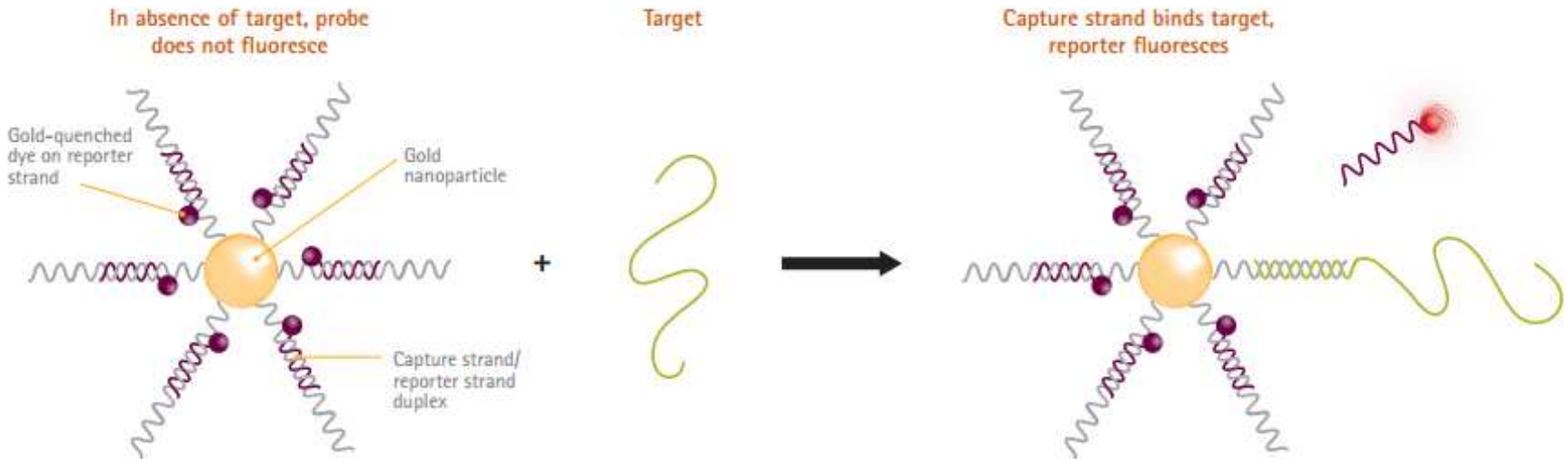
# Quantification of RNA expression

- Prime Flow® (affimetrix)
  - Fluorescent In Situ Hybridization (FISH)
    - Fixed / permabilized cells
    - A647 / a488 / a750



# RNA expression

- Smart Flare® (Merck Millipore)
  - Endocytosis of gold nanoparticles
    - Live cells
    - Cy3 (a546) or Cy5 (a647)



# RNA expression

- Advantages
  - Potential for defining GMP cell product
    - Minor sub-populations
    - Activation status
    - Potency
    - Lineage restriction
  - In-process quality control
  - Specifications

- Inconvenients
  - Limited to detection of 3 RNA per cell at one time
  - GMP grade materials
    - For information only
    - Release criteria
  - Assay validation

# Mass cytometry - CyTOF

- TOF (Time-of-Flight) based on:
  - Electric field with defined intensity and length
  - Metal ions of different mass (isotopes)
  - Detector
  - Heavier ion = longer TOF
  - Lanthanide elements (rare metal)
  - Antibodies labels with metal isotopes

# CyTOF

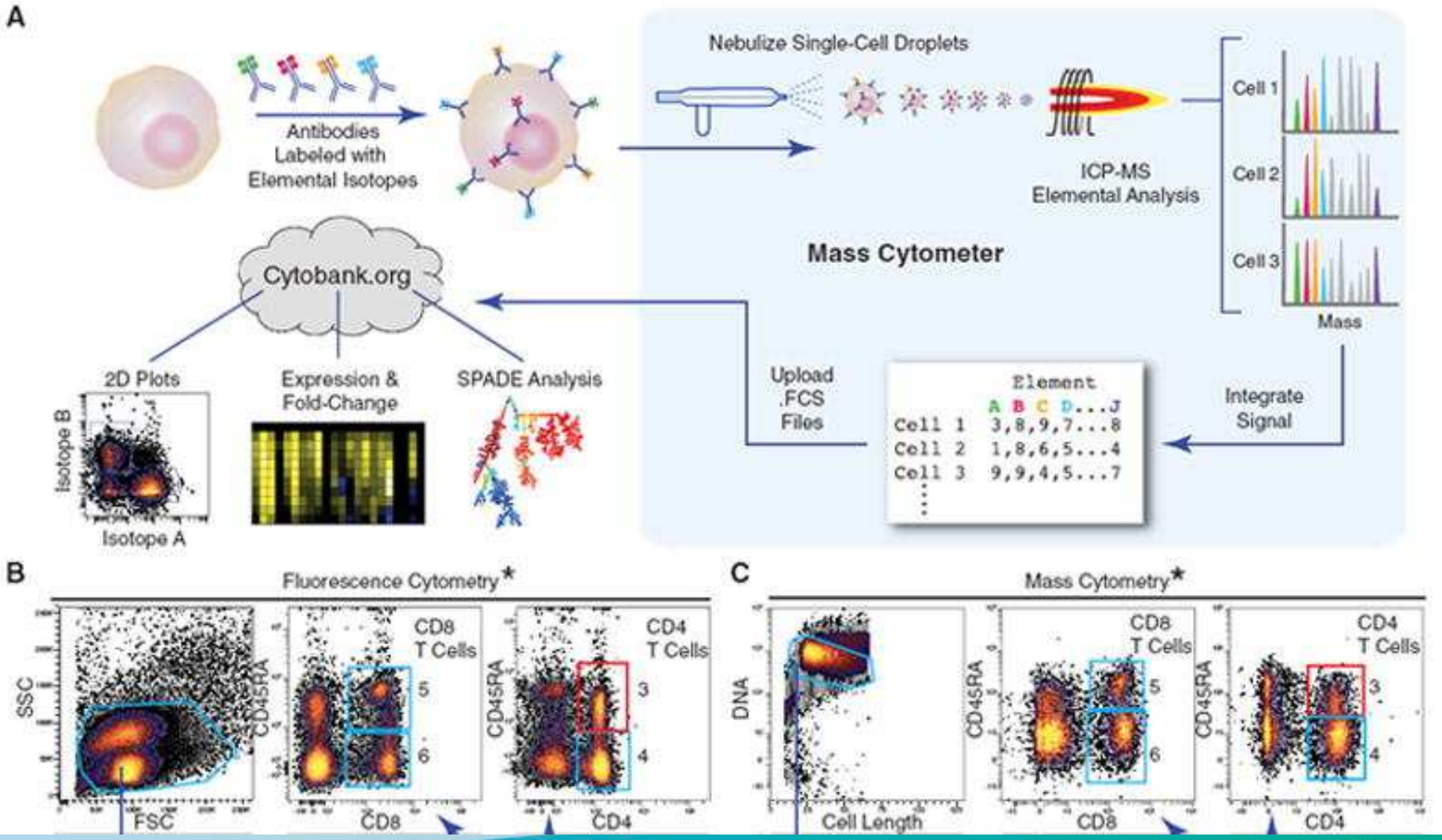



*Figure from Bendall et al. Science 2011*

# CyTOF

- Advantages
  - No spectral overlap
  - No ‘auto-fluorescence’
  - Increase number of parameters (35)
  - Quantitative
  - Well defined phenotype: signature card / barcode

- Inconvenients
  - No equivalent of SSC/FSC
  - No cell recovery
  - Low cell efficiency (80% cell lost)
  - Slow acquisition (double time/flow)
  - Limited commercially available labelled Ab
  - GMP grade reagents/software

# Conclusions

- GMP cell product characterization
  - Well defined process
  - New tools will help defined product
    - Automatic gating
    - RNA expression
    - CyTOF signature card
  - Quality improvement of cell-based therapies

thank you

cell therapies