Counting Cells.

CCU/CCQM workshop. The Metrology of quantities which can be counted

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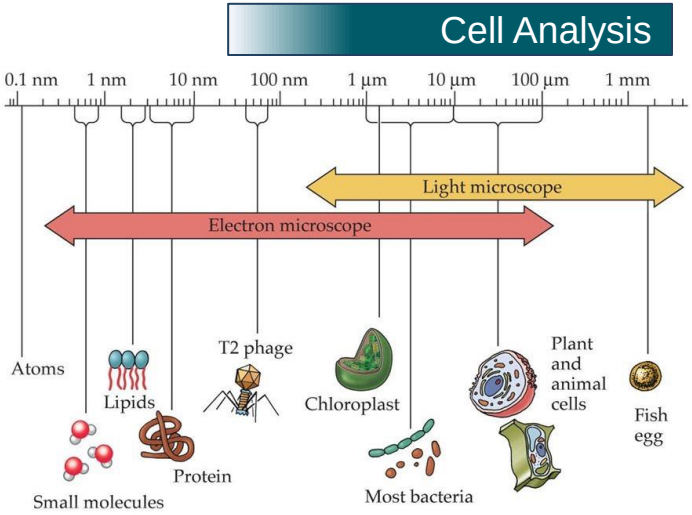
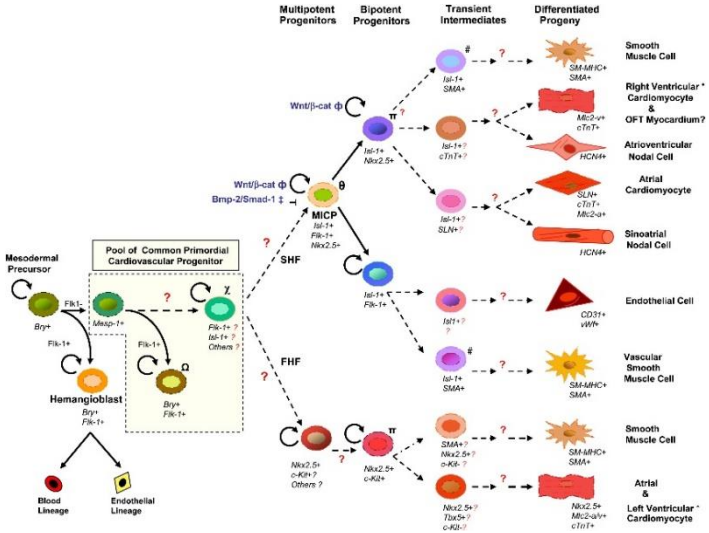
Biological complexity

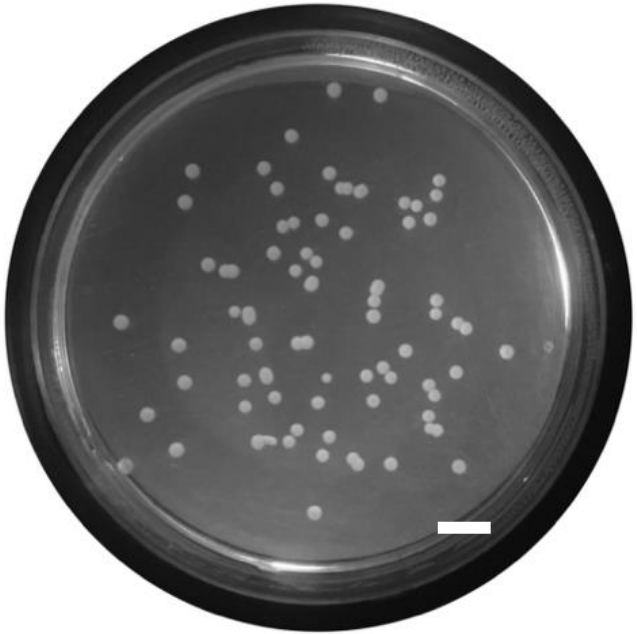


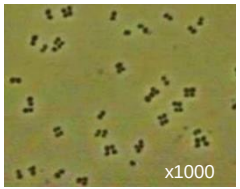


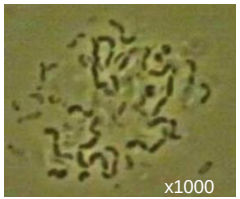
Biological complexity. Characterisation

Classical counting methods

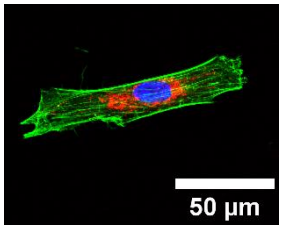
Prokaryotes

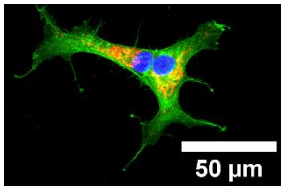


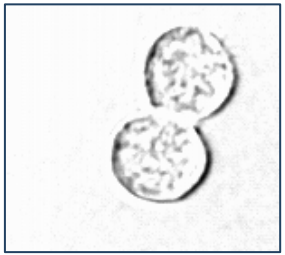


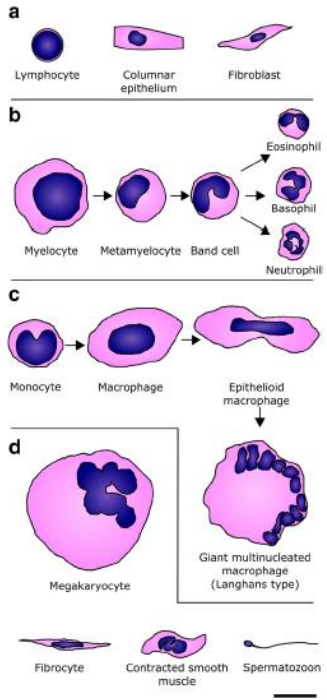


Eukaryotes





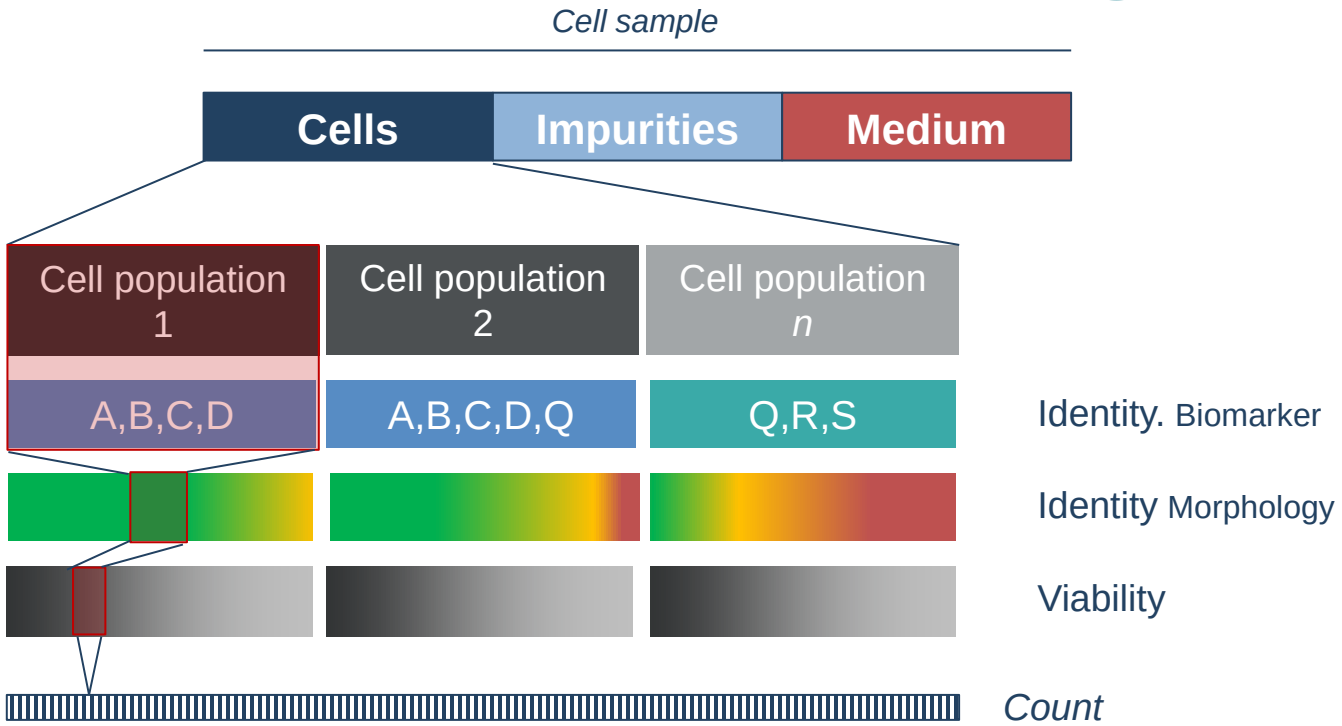




Rodrigues et al. Bioecngineering 9(7) 2022

Skinner&Johnson. Chromosoma 126:195- (2017)

Biological complexity. Characterisation



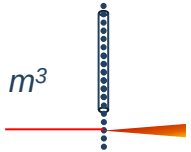
Adapted from ISO 23033:2021

Considerations for cell counting

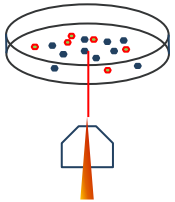
Label-free

Manual Count
Automated microscopy
Impedance counter
Flow cytometry

V. m³

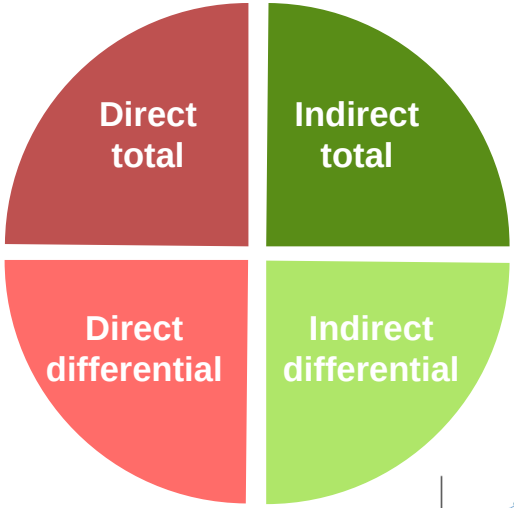


l. m

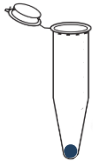


Labelling

Flow cytometry
Impedance counter (size)
Mass Cytometry
Automated microscopy
Colony forming unit

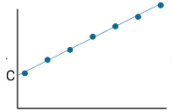


Automated microscopy
Dry cell mass/weight
Packed Cell Height
Spectrophotometry (i.e. Turbidity)



m. *Kg*

V. m³



Fluorimetry / luminometry
Spectrophotometry
Cell-based ELISA
Metabolic assays
Impedance-based biomass monitor

Single Cells (and Populations)

Populations

More sensitive to cellular preparation. ie. cell dispersal, debris

Adapted from IS 20391-12017

Quantities relating to cell counting

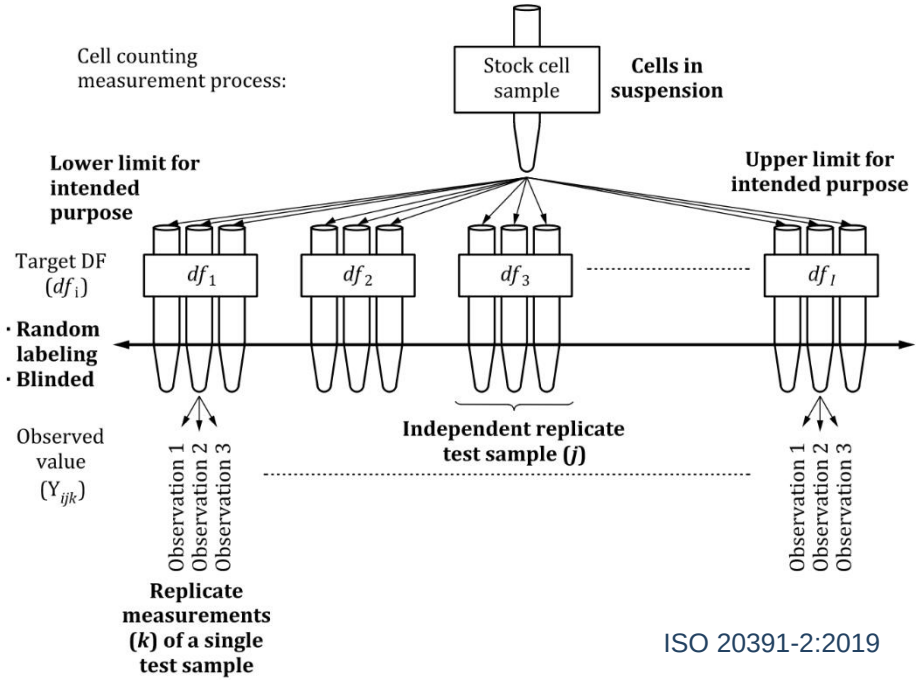
Quantity	Other metric	Expression	Description	Application (example)
Total cell count		$number\ of\ cells$ [unit 1]	Count of cells Count of events	
Differential cell count (subset of cells of interest)		$number\ of x\ cells$ [unit 1]	Count of x cells Count of x events x = nominal property	
Cell (suspension) concentration		*number of cells / volume* *number of x cells / volume* *[*unit count value/mL*]*	Cell count per volume	Multiple / Diagnostic (viable cell concentration) (AIDS <200CD4+ cells/mL)
Differential cell index or fraction		*Number of x cells/Total number of cells*	Decimal Fraction	Multiple / Diagnostic % viability % mitotic index
Packed cell volume		*Height of PCV/Height of Plasma x 100*. [%]	Fraction	Rapid diagnostic (Estimate of RBC fraction in whole blood)
Cell area density		*number of cells / area* [unit count value/mm^2*]*	Cell count per unit area	Discriminative Cell identity / morphology

Quantities relating to cell counting

Quantity	Other metric	Expression	Description	Application (example)
Average cell area		$\left(\frac{area\ occupied\ by\ cells}{number\ of\ cells}\right)$ [µm^2]	2D area of cell	Discriminative Cell identity / morphology functional response
Cell confluency		$\left(\frac{area\ occupied\ by\ cells}{area}\right) x100$	Area of an adherent population of cells covering a substrate	Cell growth kinetics. Cell manufacture (Estimate of cell proliferation 2D substrate) (Optimized sub-culture)
	Colony Forming Unit / volume	$\frac{no.of\ colonies \times dilution\ factor}{Vol.culture\ plate}$	Group of growing cells visible at micro/macroscopic scale	Estimate proliferative viable cells *a. Bacterial contamination (ProK)* *b. Stem cell number (EuK)*
	Plaque forming units / volume	$\frac{no.of\ colonies}{Vol.infectious\ lysate \times dilution\ factor}$	Group of changed or absent cells in a confluent sheet of cells	Estimate number of infectious viral particles
	Turbidity	*OD600*	Absorbance measurement of a cell suspension at 600nm.	Correlate to Bacterial/Yeast cell number. (Proliferation in a bioreactor)

Dilution series considerations.

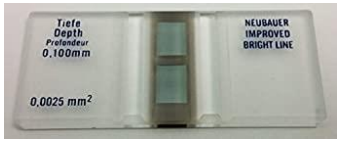
- Enable practical handling of the material to enable counting
- Present an optimal concentration to the measurement device
- Processing steps for a dilution series should avoid damaging cells
- Homogeneity of stock and replicate dilutions will be dependent on properties of cell type
- The proportionality of a cell count can serve as an internal reference to the quality of a counting process

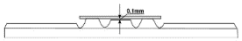


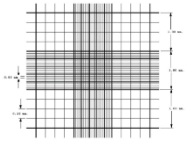
ISO 20391-2:2019

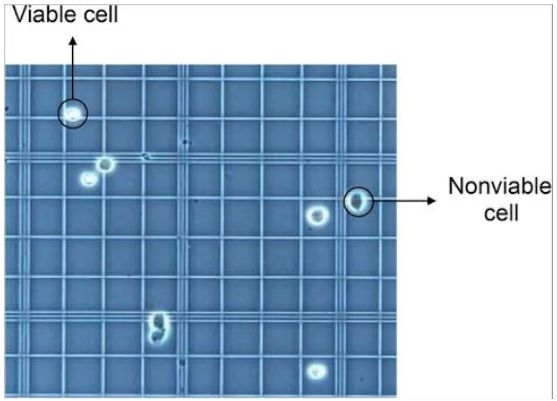
Manual counting.

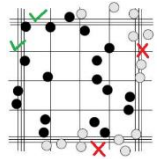
- **Operator decides on the basis of morphology or cell ultrastructure**
- Training.
  - Counting process execution (subjective recognition or cell vs. artifact)
  - Measurement process execution (pre-analytical/analytical/post analytical phase execution)
- Subjective thresholds (inclusion/exclusion criteria)
  - Dye / label selectivity/concentration
  - Cell boundary criteria
- Coincidence loss.

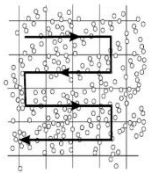






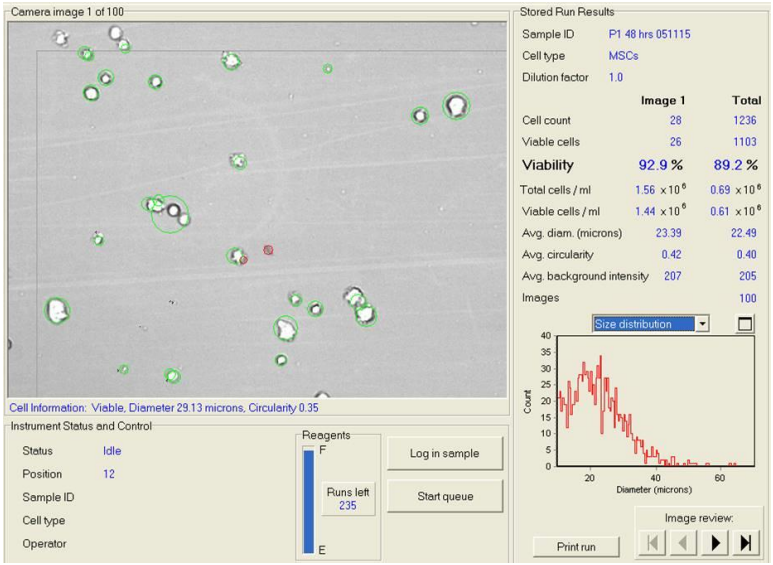






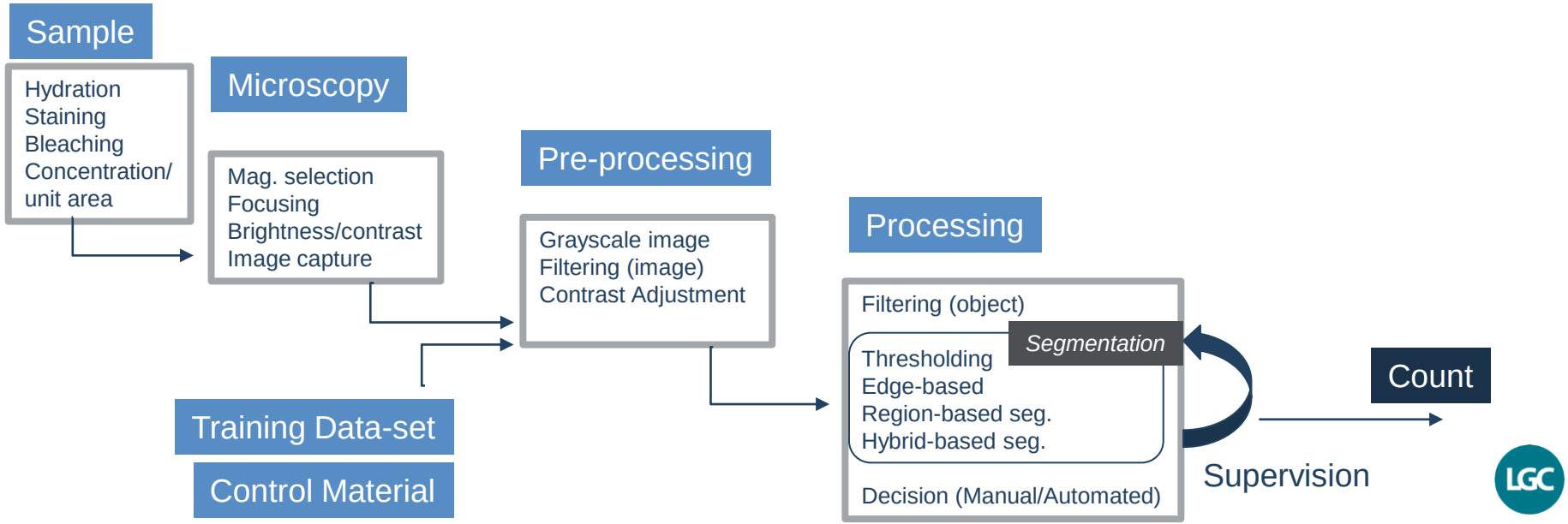
Automated image-based.

- **Image Cytometers**
  - Disposable slides or vials with different degrees of control over processing steps (ie. dye mixing, sample cueing)
  - Basic algorithms for cell identification typically based on;
    - Cell Brightness (%) (Dark to bright transition at cell boundary)
    - Cell sharpness (Arbitrary). ‘Clarity’ of edge
    - Central spot Brightness / Area. Brightness at the centre of the ROI. % of greyscale range
    - Minimum Circularity
    - Decluster degree
  - Output typically histogram Count / Size

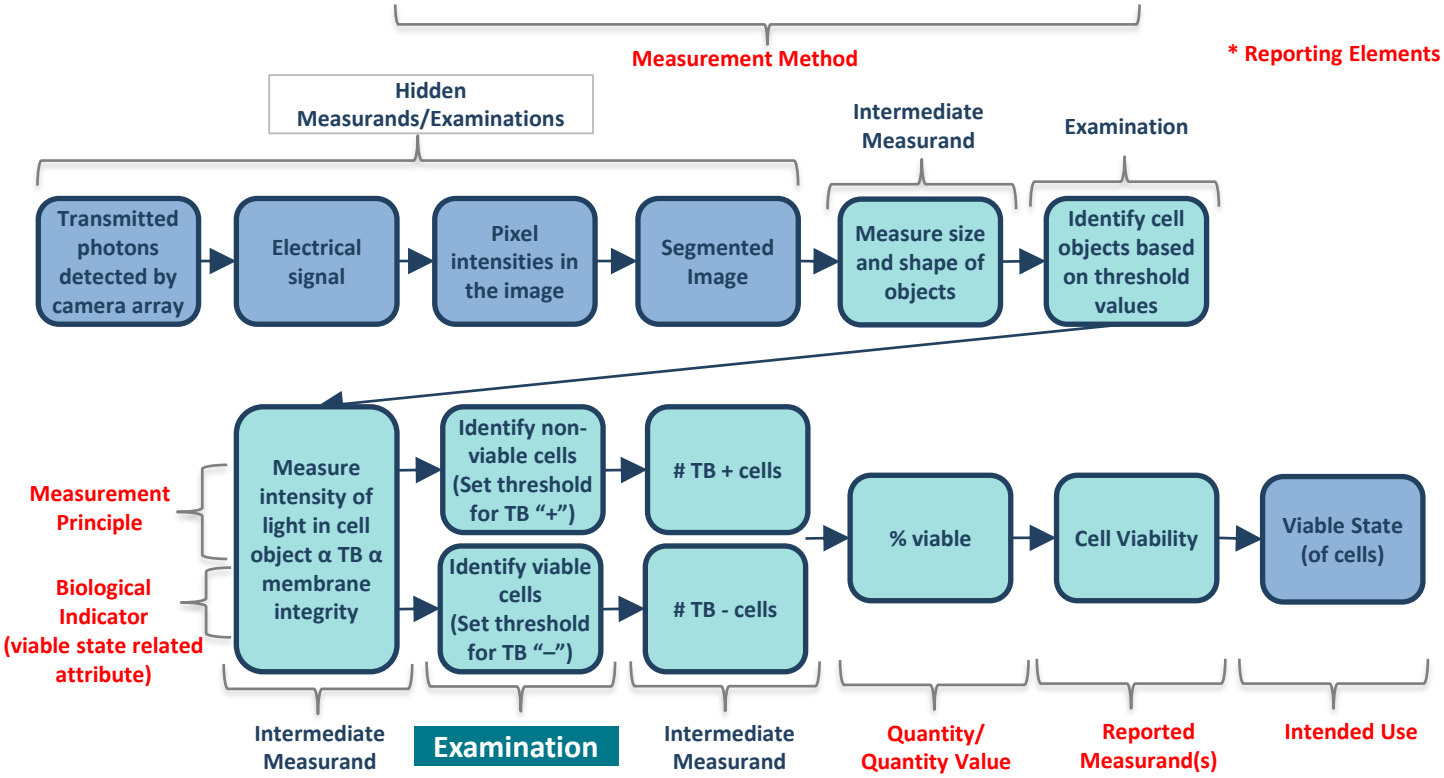


Automated image-based.

- **Computational image-based cytometry**
  - Differentiate cells by features beyond what is possible by observation
  - Development of robust machine-learning based algorithms, with different level of supervision

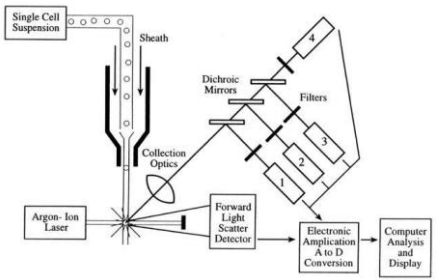


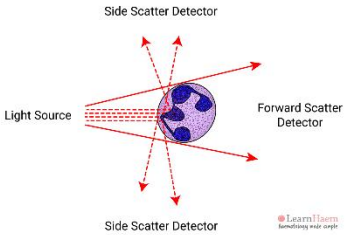
Measurand Chart: Image Based Trypan Blue Dye Exclusion Test for Cell Viability

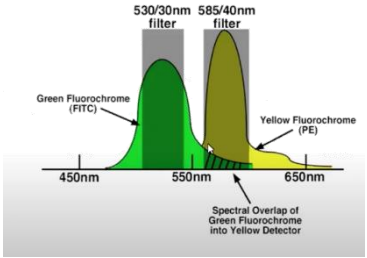


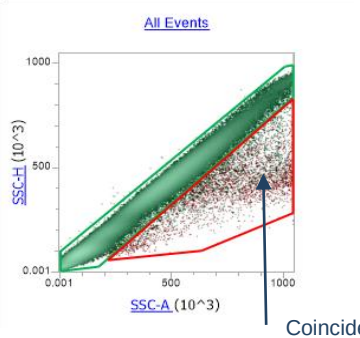
Flow cytometry

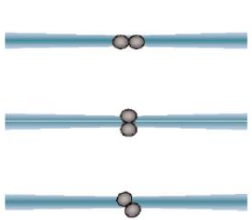
- **Polychromatic flow cytometry**
- Optical. Laser/LED light scattered by cells/particles and detected as events
- Multi-parametric information at single cell level
- Only singlets typically used for onward analysis
- Subset of total events forms the analytical data
  - Subjective manual gating or automated gating.
- Only a portion of fluorescence information is collected

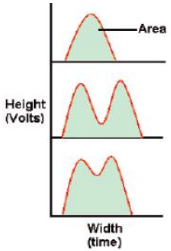












Coincidence loses

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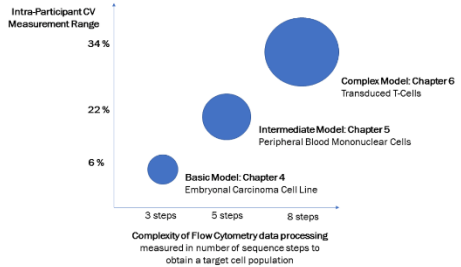

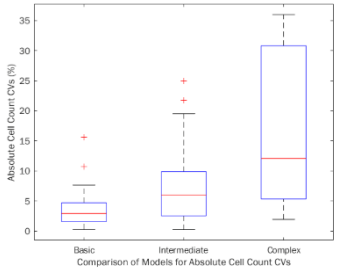

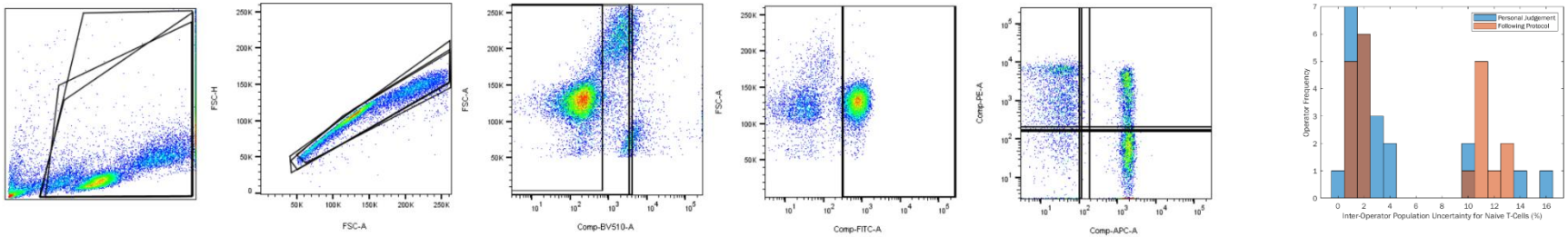

Rebecca C Grant. PhD thesis 2019. Univ. Loughborough

Flow cytometry

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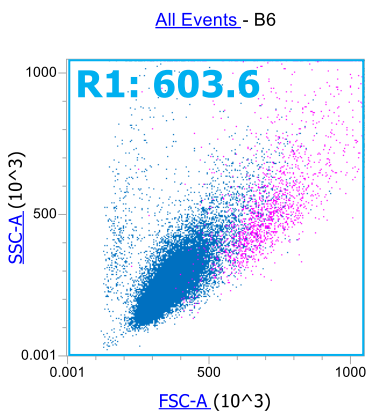
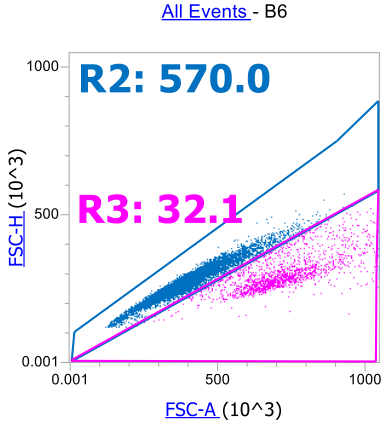
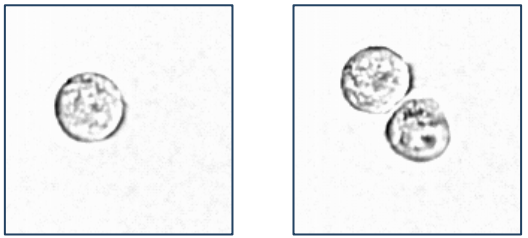


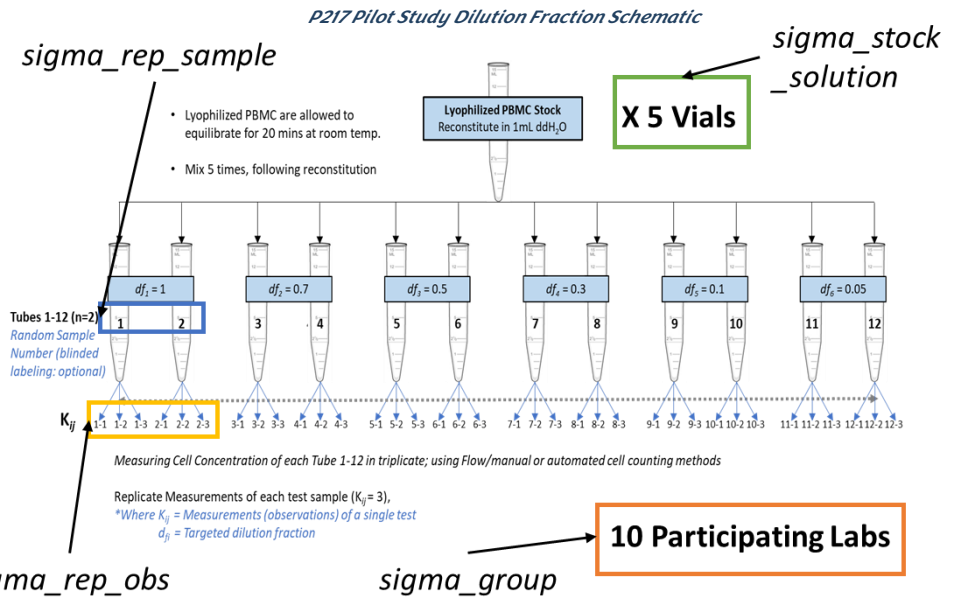


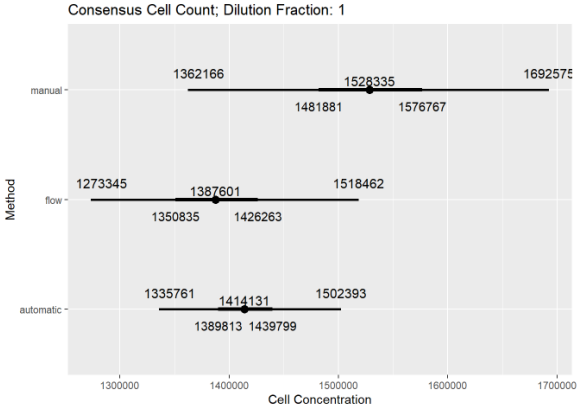
- **Imaging Flow cytometry**
- Brightfield or fluorescence images obtained for each event.

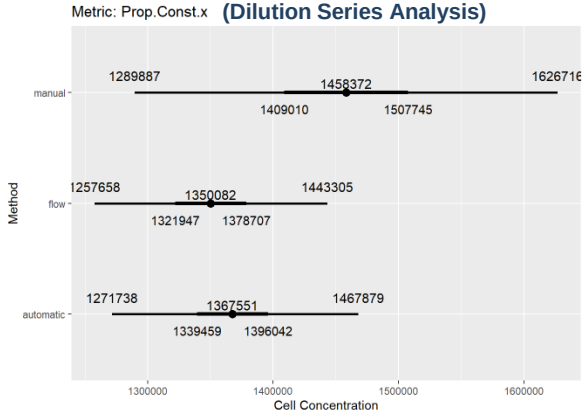


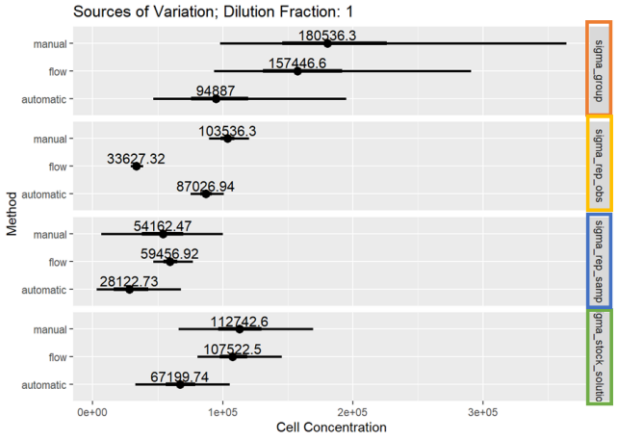
P217 Pilot Study: Enumeration of fixed peripheral blood mononuclear cells in suspension

- **Measurement Claim: Quantification of absolute cell numbers in a PBMC preparation**
- **Evaluate a lyophilized, fixed preparation of peripheral blood mononuclear cells (PBMCs) as a comparator for manual, automated and flow cytometry-based cell counting methodologies (Consensus Value/Sources of Variation)**
- **Uses a wide range of cell counting technologies together with a prescribed dilutions series design adapted from ISO 20391-2**
  - **Flow Methods – Detailed SOP**
  - **Manual Method – Some guidelines but in-house protocols were followed**
  - **Automated Methods – no guidelines; include any**









Further considerations

- **Counting for entities that are fundamentally different**
- **The place of examination within the counting process is critical**
  - An operator decides on the basis of morphology or cell ultrastructure (Manual counting)
  - An automated count relies on predefined limits (operator) or training set quality
- **What is the status of the measurand for cell counting?**
  - How do different measurands relate in complex entity quantification?
- **The assignment of value to a cell and cell property.**
  - Nominal viability properties (live/dead) versus ordinal binary (ie.1/0)
- **The place of digital specifications for control in cell analysis**
  - How do we address the stability issue for biological entities?

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