Reference Controls
Requirements for Optimal Reference Controls

• Needed to calculate spillover (slope) between fluorochromes

• How to get an accurate calculation?
  • The more separate the two data points are, the better the calculation
    • Bright particles are necessary for this
  • Both particles need to have IDENTICAL autofluorescence characteristics
    • If negative particles are beads, then the positive particle need to be the exact same beads (same lot)
  • There is need to have enough events for both data points
    • Stopping rules need to be adjusted according to the sample type and marker used
  • The fluorescence spectrum of the positive data points needs to be IDENTICAL to the one in the multicolor sample
    • Special considerations when using tandem dyes
    • The spectrum of the reagent binding to beads may be different to the spectrum of the same reagent when bound to cells!
Reference Controls: Making Good Choices

• **Should I use beads or cells as controls?**
  • Beads are easy to use and it is very likely that they will have a bright positive signal. It’s also easy to collect enough events.
  • HOWEVER, users need to assess whether the spectrum of the reagents used to stain the beads matches the one when stained on cells
    • If possible compare unmixing results using beads vs cells as reference controls
  • Users also need to assess how forgiving a specific assay is if there are errors in the calculations

• **I want to use cells, but my marker is rare or very dim. What can I do?**
  • If a fluorochrome is NOT a tandem, replace with a marker highly expressed in a distinct population (CD3, CD4, CD8, B220 etc).
    Example: instead of using CD25 PE, use CD4 PE.
  • If fluorochrome is a tandem, only option is to use beads stained with exactly same reagent (same lot)

• **Can I use cells as negative control for all my controls?**
  • Only when ALL controls are also cells (same cell type, same sample prep procedure)
Unstained Control vs Negative Reference Control Population

- **In addition to the Reference Controls, an Unstained Control is mandatory for Spectral Unmixing**
  - This control is NOT needed for spillover calculation
  - This control is used for measurement of autofluorescence assessment and extraction
  - This control needs exactly match the particle type and sample prep procedure used in the multicolor samples
  - Current software version only allows for one unstained control

- **My Reference Control does not have a negative population. Can I use the unstained control for spillover calculation (universal negative)?**
  - Only if the reference controls are the same cell type / sample preparation procedure as the unstained control
  - Due to current software limitation, best workflow is to have reference controls that have a negative and positive population in each tube