# Flow Experiments Come Alive

Featuring ZE5™ Cell Analyzer, Bio-Rad Antibodies and Reagents

BIO-RAD

# Practical Advice for Common Flow Assays

Flow cytometry is a powerful technology used in a wide variety of biological research. Here we will walk you through common flow assays, with examples, key facts and top tips to help your research. See how your flow experiments can come alive with high quality antibodies, reagents and the ZE5™ Cell Analyzer.

| Assays | | Reagents |
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| Cell Viability | p3 | Non-fixable DNA binding dyes and fixable protein binding dyes |
| Immunophenotyping | p4 | Conjugated and unconjugated flow validated antibodies against immunology markers |
| | | Conjugation kits |
| | | Buffers for staining, fixation, washing and permeabilization |
| Apoptosis | p6 | Kits, antibodies and reagents for detection of early, mid and late apoptosis |
| Proliferation, DNA and Cell Cycle Analysis | p8 | Antibodies, DNA dyes and cell permeable dyes |

**Instrument**

## ZE5 Cell Analyzer

Superior performance and flexibility to align with your flow cytometry needs.

Explore more at **bio-rad-antibodies.com/ZE5**

# Viability Staining

Identification of viable cells is vital in many cell assays. Toxicity or side effects of drugs or treatments can affect viability. Therefore sample viability is important to ensure reproducibility of your experiments and confirm that you are studying a live population. The presence of dead cells can greatly affect the quality of your data in flow cytometry as they have increased autofluorescence and non-specific antibody binding, potentially leading to false positives and increased background noise (Figure 1). This background may make weakly positive populations harder to identify.

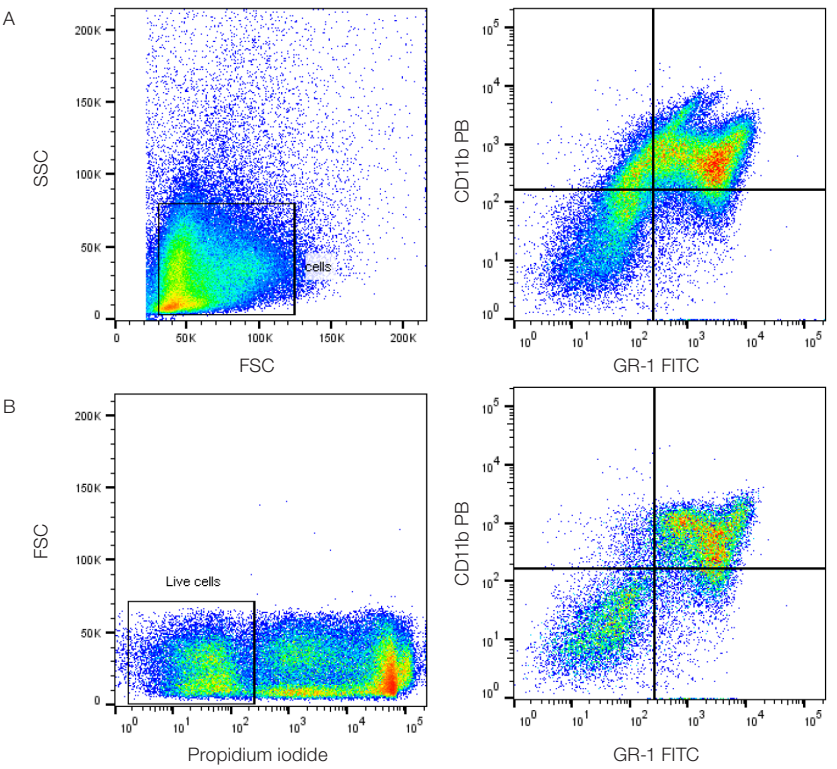


**Fig. 1. Use of a viability dye to improve staining. A**, use of forward and side scatter may not be sufficient to remove dead cells. **B**, dead cell exclusion using ReadiDrop™ Propidium Iodide (cat. #1351101) allows easier identification of positive and negative populations. Images shown here are mouse bone marrow stained with Rat Anti-Mouse CD11b Pacific Blue (#MCA711PB) and Rat Anti-Mouse GR-1 FITC (#MCA2387F). Data acquired on the ZE5 Cell Analyzer.

### Key Facts

- Improve your data
- Easy-to-use
- Offer wide selection of available dyes
- Available in various excitation and emission spectra

### Top Tips

- Use instead of forward and side scatter gate to remove dead cells
- Use in all flow experiments
- Do not fix DNA dyes

# Immunophenotyping

Analyzing the presence and frequency of specific cell populations, in normal versus malignant samples, or in response to treatments is the most common and well known use of flow cytometry in basic research and clinical labs. Cells from virtually any source can be identified by markers, using specific antibodies conjugated to fluorophores. Staining with multiple antibodies, conjugated to different fluorophores with specific excitation and emission profiles, allows simultaneous detection and quantification of many markers. This allows many different cell populations to be identified in a single sample (Figure 2). Panels have become more complex to now include a combination of surface and intracellular staining, activation state and cytokine release.

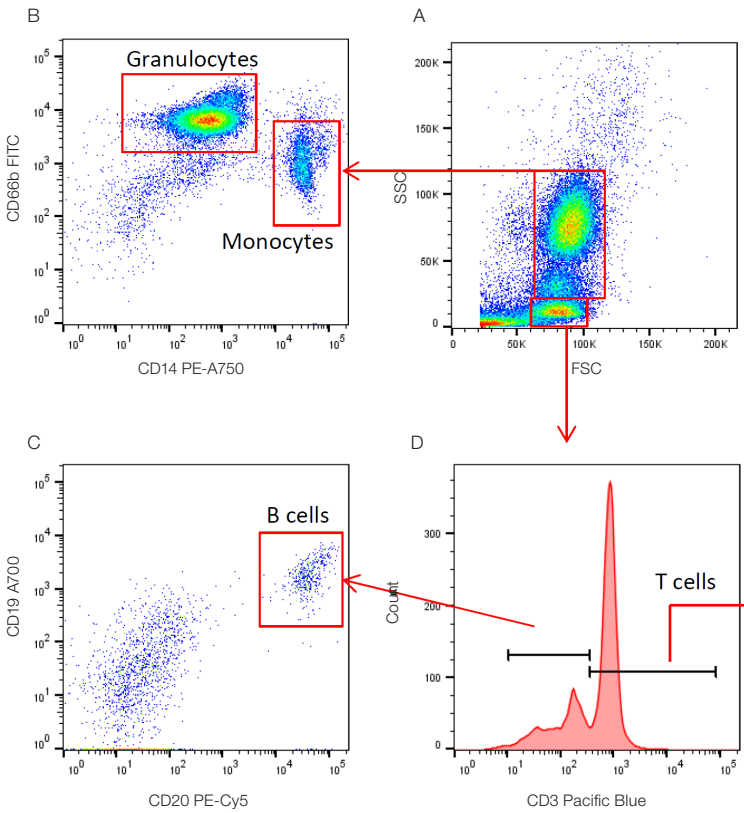


**Fig. 2. Common leukocyte panel with gating strategy.** Seven color panel of CD3, CD4, CD8, CD19, CD20, CD14 and CD66b to identify T cells, B cells, monocytes and granulocytes in human peripheral blood. **A**, cells were gated using forward and side scatter. **D**, T cells were identified by CD3 expression. **E**, helper and cytotoxic subsets were identified by the expression of CD4 and CD8 respectively. **C**, B cells were identified as $CD3^-$, $CD19^+$ and $CD20^+$. **B**, the myeloid populations were able to be further identified as $CD14^+$ monocytes, and $CD66b^+$ granulocytes. Data acquired on the ZE5 Cell Analyzer.

## Key Facts

- Characterize multiple cell populations using the 30 available parameters on the ZE5 Cell Analyzer
- Isolate multiple cell types with sequential gating
- Identify fluorescent proteins and transfected cells
- Combine surface and intracellular staining
- Improve assays by using our specialized buffers

## Top Tips

- Ensure good sample prep
- Use a viability dye
- Choose the right antibody clone
- Titrate your antibody
- Consider the fluorophore brightness, excitation and emission
- Know the biology of your sample
- Use controls

# Antibody Conjugations

Not all antibodies are available directly conjugated to a fluorophore. One alternative is to use a secondary antibody. Whilst this can give useful amplification of signal, non-specific binding and number of controls will also increase and the extra washing steps may result in loss of precious sample. Multiplexing can be a problem if the primary antibodies are from the same species, or the same isotype. Solve this by using a conjugation kit, a quick and reliable method to conjugate a wide range of fluorophores to antibodies.

## LYNX Conjugation Kits®

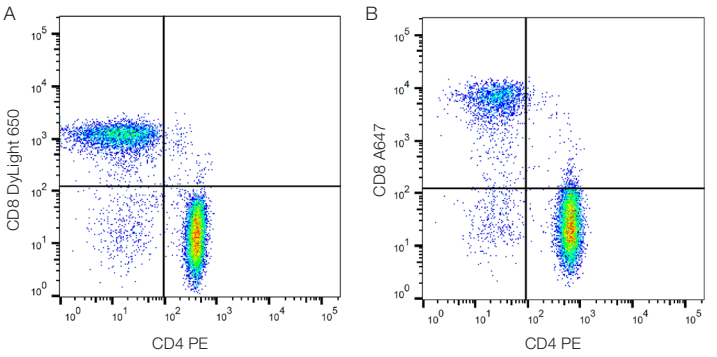


**Fig. 3. Comparison of LYNX Kits with directly conjugated antibodies.** **A**, purified CD4 (#MCA1267) and CD8 (#MCA1226) were labeled with LYNX Rapid RPE Antibody Conjugation Kit (#LNK021RPE) and LYNX Rapid Plus DyLight 650 Antibody Conjugation Kit (#LNK241D650) respectively, and used to stain human peripheral blood. **B**, Alternatively directly labeled antibodies were used. The staining shown is lymphocytes gated on the CD3 positive population. CD4 and CD8 positive T cells can be identified in both plots. Data acquired on the ZE5 Cell Analyzer.

## ReadiLink Conjugation Kits

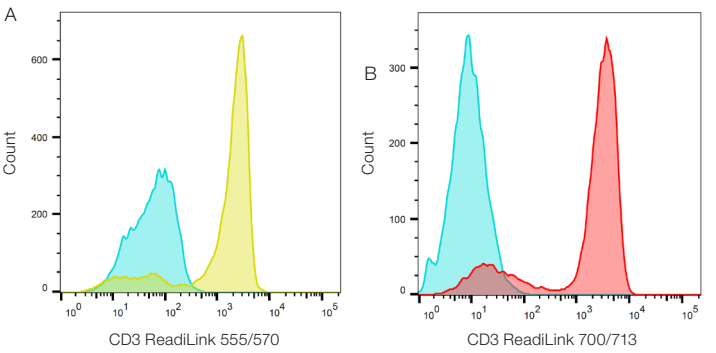


**Fig. 4. Staining using ReadiLink Kits.** Unconjugated CD3 antibody was incubated with **A**, ReadiLink 555/570 Kit (#1351003) or **B**, ReadiLink 700/713 Kit (#1351008) to create conjugates. After addition of the quencher the antibody was ready to use. Peripheral blood was stained with the freshly conjugated antibody. Distinct T cell populations were visible without the use of secondary antibodies, with good separation of the positive and negative populations. Data acquired on the ZE5 Cell Analyzer.

### Key Facts

- Conjugate microgram to milligram amounts of antibody
- Available in a wide range of fluorophores
- Eliminate need for a secondary antibody
- Reduce number of wash steps
- Minimize hands-on-time
- Ready in an hour

### Top Tips

- Consider the fluorophore brightness, excitation and emission
- Know the biology of your sample
- Avoid sodium azide
- Use ReadiLink Kits for flow and LYNX Kits for larger scale conjugation

**Think Antibodies. Think Bio-Rad.**

# Apoptosis Assays

Flow cytometry is crucial in the analysis of cell populations to determine levels of programmed cell death, or apoptosis. This highly regulated mechanism for selectively eliminating cells without the inflammation associated with necrosis, plays an important role in embryogenesis, maintaining organ size and removal of damaged or aberrant cells. The importance of apoptosis is underscored by the many diseases such as neurological and cardiovascular disorders, autoimmune diseases and cancer resulting from dysregulation of this process.

Apoptosis can be initiated through the intrinsic and extrinsic pathways and follows distinct signaling pathways (Figure 5). The identification of the pathway and which mediators are activated can be important considerations when studying apoptosis as it may help determine a disease mechanism or allow intervention and potential therapy. Furthermore when combined with immunophenotyping (p4), specific cell subsets undergoing apoptosis can be identified.

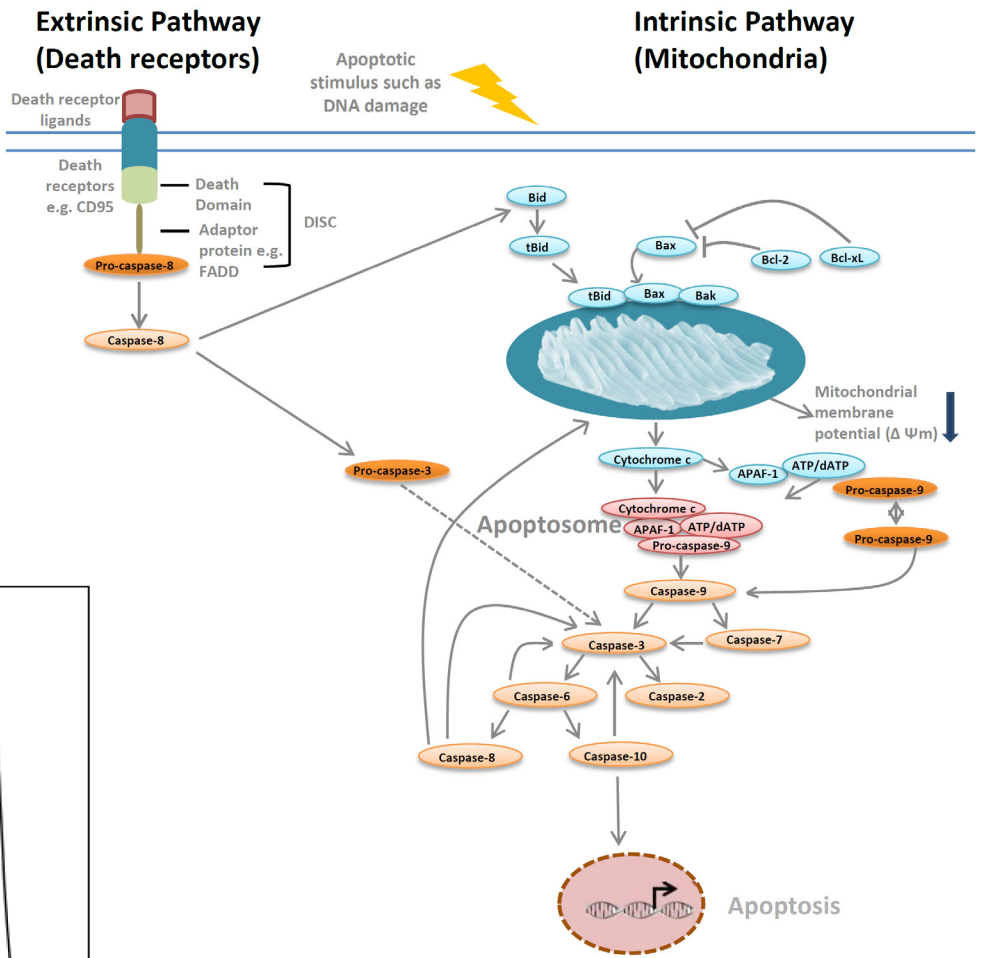


**Fig. 5. Overview of the extrinsic and intrinsic apoptosis pathways.** Key components of the apoptosis pathways from stimulation, removal of survival signals, loss of mitochondrial membrane potential and caspase activation.

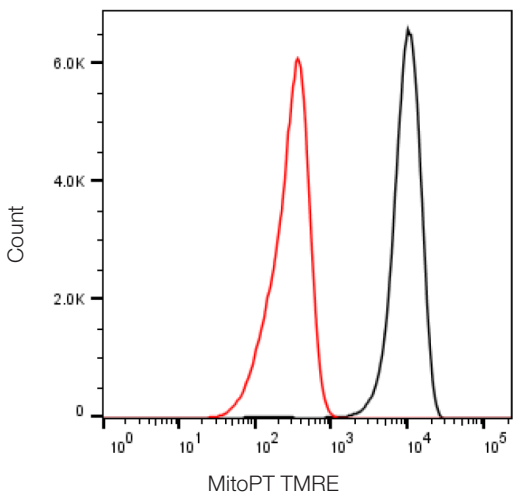


**Fig. 6. Loss of mitochondrial potential during apoptosis.** Jurkat cells were treated with CCCP at 50 µM to induce apoptosis. A loss of membrane potential is reflected by the loss of fluorescence staining from the mitochondria as the dye escapes due to membrane disruption, using MitoPT TMRE Kit (#ICT946). Data acquired on the ZE5 Cell Analyzer.

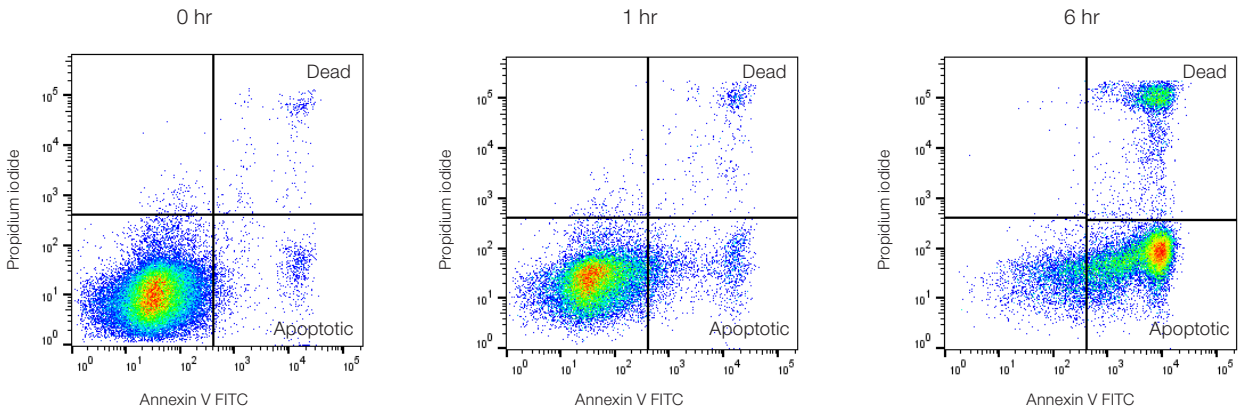


**Fig. 7. Annexin V detection at the cell surface during apoptosis.** Jurkat cells were treated with staurosporine at 1 µM to induce apoptosis. The cells were then stained with annexin V FITC (#ANNEX300F) and ReadiDrop™ Propidium Iodide (#1351101). Apoptotic cells positive for annexin V can be seen in the bottom right quadrant and dead cells positive for both annexin and PI in the top right quadrant. Healthy cells are negative for both stains. Data acquired on the ZE5 Cell Analyzer.

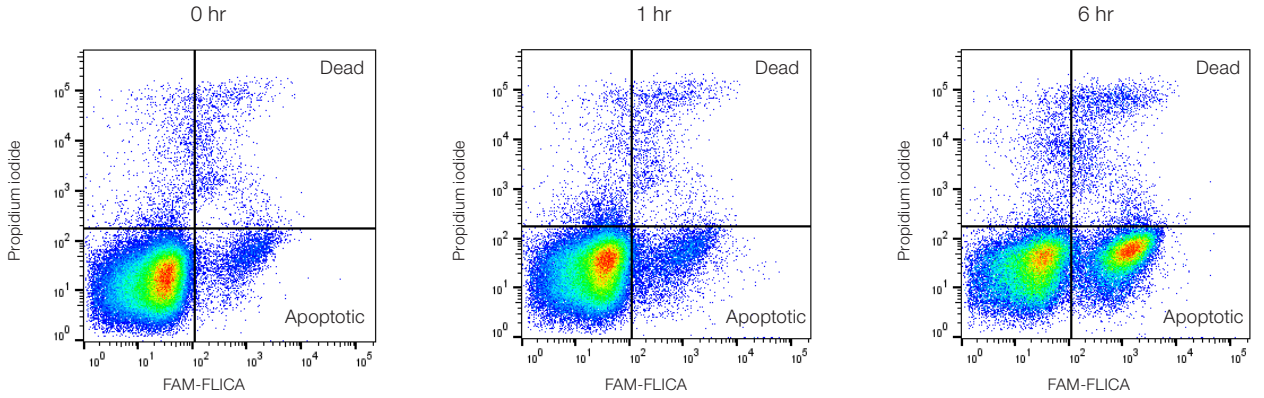


**Fig. 8. Caspase activation during apoptosis.** Jurkat cells were treated with staurosporine at 1 µM to induce apoptosis. Active caspases can be detected using FAM FLICA Poly Caspase Kit (#ICT091) after 6 hr of treatment. Data acquired on the ZE5 Cell Analyzer.

## Key Facts

- Distinguish between intrinsic and extrinsic pathways
- Different assays for early, middle and late apoptosis
- Easy-to-use kits available
- Apoptosis needs to be identified from necrosis, pyroptosis, necroptosis and autophagy
- Compatible with immunophenotyping

## Top Tips

- Include vehicle alone and positive controls
- Use a viability dye
- Combine two assays to confirm apoptosis
- You may still need to compensate
- Alter your gating strategy to include shrinking cells
- Cell lines may act differently from primary cells

# Proliferation and Cell Cycle Assays

## Proliferation Assays

Cell activation and proliferation, determined by marker upregulation, calcium signaling or phosphorylation, provide an effective method to determine immunocompetence and cell reactivity. Alternatively quantitative methods to determine proliferation in cells include using cell labeling dyes such as CytoTrack™ Assays (Figure 9) or incorporating BrdU, a thymidine analog, into the DNA of proliferating cells.

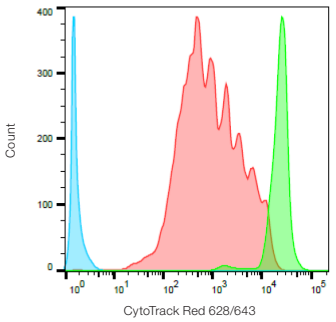


**Fig. 9. Proliferation of human peripheral blood lymphocytes.** Human PBLs were stained with CytoTrack Red 628/643 Cell Proliferation Kit (#1351205) and stimulated for 5 days with PHA. Cells that have proliferated show a reduction in the amount of dye with each cell division. Stimulated cells (in red), labeled cells but unstimulated (in green) and unlabeled cells (in blue). Data acquired on the ZE5 Cell Analyzer.

### Key Facts

- Combine with surface markers
- Does not give effector function

### Top Tips

- Include viability dyes
- Use maximal and unstimulated controls

## Cell Cycle Assays

Cell cycle phases, anomalies, checkpoints and DNA damage are identified using DNA dyes. The amount of cells in $G_0$, $G_1$, S and $G_2$ can be determined as cells in $G_2$, which have twice as much DNA as cells in $G_1$, will have double the fluorescence (Figure 10). The presence of cells that have >2N amounts of DNA and sub $G_1$ is indicative of cellular transformation and apoptosis respectively.

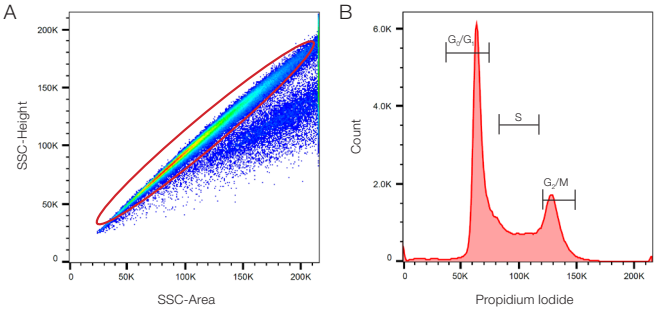


**Fig. 10. Jurkat cells were fixed in 70% cold ethanol, treated with RNAse and stained with PI to reveal the stages of the cell cycle.** Doublets were excluded in A, prior to analysis in B. Data required on the ZE5 Cell Analyzer.

### Key Facts

- Ethanol fixation needed
- RNA binding dyes require cells to be treated with RNAse

### Top Tips

- Long term ethanol storage can improve staining
- Slow speed acquisition can improve data

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