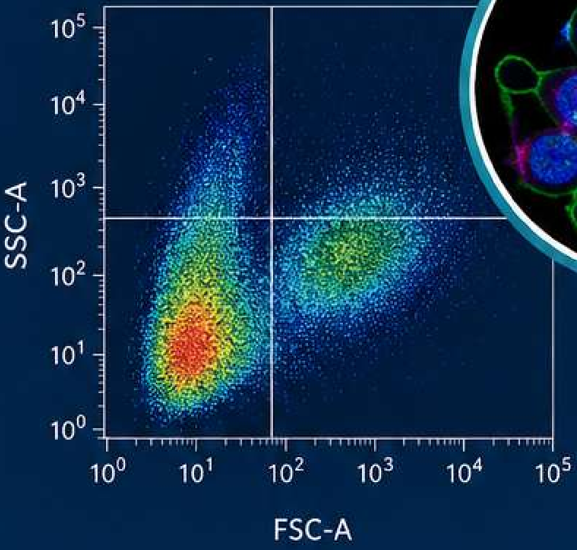
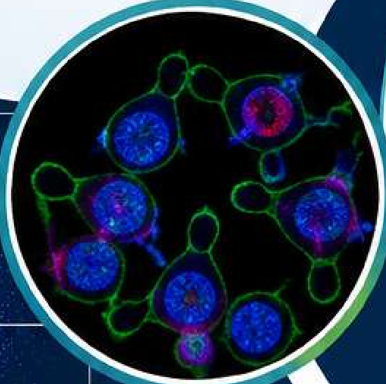
Guidelines for the use of

Flow cytometry (FACS)

in cell biology & immunological studies





ACCURATE ANALYSIS

RELIABLE RESULTS

APPLICATIONS IN CELL BIOLOGY & IMMUNOLOGY

BEST PRACTICES FOR QUALITY DATA

Better Data. Better Insights. Better Science.

Guidelines for the use of flow cytometry and FACS in immunological studies

Flow cytometry, also known as, flow-assisted cell sorting (FACS) has become one of the most powerful technologies in modern immunology. It enables researchers to rapidly analyze millions of individual cells, identify immune cell populations, evaluate cellular function, and isolate specific cells for downstream analysis. However, obtaining reliable and reproducible data requires far more than sophisticated instruments—it depends on standardized experimental design, proper sample handling, rigorous quality control, and consistent data analysis.

Scientists from across the world has presented one of the most comprehensive international references on flow cytometry. Their publication provides a consolidated expert recommendations from leading immunologists worldwide to establish standardized methodologies that improve research quality and reproducibility.

Key Recommendations before initiation of FACS

1. Experimental Design and Planning

- Clearly define the biological question and aim of the experiment to appropriately select the antibody panels. Implementation of standardize protocols across all the samples ensures minimal variability.
- Relevant biological controls are essential as a part of good flow cytometry practice.
- Every experiment should contain three fundamental categories of controls, namely setup

- **Instrument controls**, particularly standardized fluorescent beads, are used to check whether the flow cytometer is functioning consistently. They can reveal changes in laser performance, detector sensitivity, fluidics, or optical alignment.
- **Specificity/Gating controls** allow specific staining to be distinguished from non-specific staining. They prevent background autofluorescence, provides correct compensation value for the fluorochrome panel and aid in clear disguising the viable sample from the non-viables cells which may get stained non-specifically.
- **Biological controls** provide relevant biological comparisons between stimulated/treated and unstimulated/untreated cell samples. It is recommended to keep both positive and negative biological controls to determine if a treatment or condition induces a true detectable change in the observed biology of the cell population of interest.

- Ensure that the FACS instrument is properly optimized, calibrated and standardized to produce correct and reproducible results, providing confidence of any changes in the sample that are detected are due to true biological events not to instrumentation issues.
- Document all experimental conditions for reproducibility.

Practical importance: Well-designed experiments with standard controls reduce bias and improve confidence in the findings.

Control	Main purpose
Fluorescent calibration/QC beads	Check instrument performance and stability
Unstained cells	Determine cellular autofluorescence/background
Single-stained controls	Establish fluorescence spillover and compensation
FMO controls	Help determine appropriate gating boundaries
Positive controls	Confirm that the assay/staining can detect the expected response
Negative controls	Establish background or absence of the target response

2. Instrument Setup and Calibration

Proper instrument performance is essential for reproducible data.

Recommendations include:

- Selection of optimal cell sorting nozzles and sheath pressure based on cell size, cell sensitivity, desired sort speed and the manufacturer's recommended operating parameters.
- Daily quality control checks.
- Laser performance verification.
- Detector sensitivity testing.
- Standardized voltage settings.
- Routine calibration using reference beads.

- Monitoring instrument stability over time.

Outcome: Consistent results across experiments and laboratories.

3. Sample Collection and Preparation

Sample quality is one of the most important determinants of reliable results.

Best practices include:

- Process samples as quickly as possible after collection, preferably on the same day. Fresh samples generally perform better than cryopreserved samples.
- It is recommended to plan the experiment such that cell collections should start in the morning and FACS reading gets completed till evening.
- Maintain sterile handling procedures while handling the cell samples to avoid false readings due to non-specific antibody binding with the contaminant.
- Minimize mechanical stress during pipetting and freeze-thaw cycles, if samples are stored overnight.
- Remove cell clumps and debris using mild pipetting (with 1 mL pipette) before analysis.
- Most importantly, it is recommended to assess cell viability before staining, to prevent wastage of antibodies and staining dyes, if cells are dead.

Practical importance: Proper sample preparation preserves cell integrity and minimizes technical artifacts.

4. Cell Viability Assessment

Dead cells can bind antibodies non-specifically and distort results.

The guidelines recommend:

- Using viability dyes before acquisition.
- Excluding dead cells during gating.
- Maintaining high cell viability throughout processing.

Outcome: More accurate identification of live immune cell populations.

5. Cell Sorting Guidelines

For fluorescence-activated cell sorting (FACS), it is recommended to:

- Optimize device nozzle size and sheath (fluid) pressure by routine cleaning by running cleaning/decontamination cycle according to the manufacturer's protocol. Also check that the fluid stream is stable and the droplets are forming correctly. For this drop-delay calibration can be done before initiating cell sorting.
- Maintain sterile conditions by performing decontamination cycle using 70% ethanol solution and then washing the nozzles and pressure pipes using sterile buffer solution, preferably phosphate buffer saline (PBS) solution. It is highly recommended to decontaminate the instrument after sorting, particularly when working with biological samples.
- Monitor sort purity & protect cell viability to ensure that efficient selection of the desired population. This is particularly important when sorted cells will be used for culture, RNA extraction, functional assays or downstream experiments.

  For this, proper cell gating, sorting healthy population, avoiding aggregates, maintaining optimal cell storage conditions are mandatory. Higher stress the cells experience during sorting, lower is their post-sort viability.

For sensitive applications, the instrument's fluidics, nozzle/flow cell, sample line and collection area are particularly important.

Antibody Selection and Panel Design

Modern flow cytometers can measure more than 30–40 parameters simultaneously.

The BD® Spectrum Viewer | Spectral Analyzer is an online flow cytometry panel-design tool can make fluorochrome selection much easier. It helps to choose suitable fluorochromes, select compatible filters, and assess potential spectral spillover before finalizing a panel, comparing the excitation (Ex) and emission (Em) spectra across a large and expanding library of dyes and probes. In addition, it includes pre-loaded cytometer configurations, a stacked laser view, and options to save and share panel designs. For spectral flow cytometry, users can visualize and analyze fluorochrome emission profiles and use a heat map to quickly identify regions of overlapping emission.

1. Antibody selection guidelines

- Selecting antibodies with validated specificity. Also consider the antigen expression level and co-expression of markers.

- Choosing fluorochromes compatible with the instrument. For this match fluorochrome brightness to antigen density. Evaluate fluorochrome–detector combinations using spillover/spreading information.
- Avoiding unnecessary spectral overlap. Use highly separated fluorochromes for critical low-expression markers.
- Testing antibody panels before large-scale experiments. Titrate antibodies rather than assuming the manufacturer's recommended concentration is optimal.

Practical importance: Optimized panel design improves sensitivity and reduces compensation errors.

Major Fluorophore Classes Used in Flow Cytometry

Fluorophore class/family	**Common fluorophores**	**Typical excitation laser**	**Ex. max (nm)**	**Em. max (nm)**	**Typical spectral region**
Fluoresceins	FITC	488 nm	495	519–520	Green
Alexa Fluor green	AF488	488 nm	499	520	Green
Phycoerythrins	PE	488/532/561 nm	~566	~578	Yellow-orange
Tandem PE dyes	PE-Texas Red / PE-eFluor610	488/561 nm	~565	~610	Orange-red
PE-Cy5	PE-Cy5	488/561 nm	~565	~670	Far-red
PE-Cy5.5	PE-Cy5.5	488/561 nm	~565	~695	Far-red
PE-Cy7	PE-Cy7	488/561 nm	~565	~780–785	Near-IR
PerCP family	PerCP	488 nm	~482	~675–678	Red
PerCP-Cy5.5	PerCP-Cy5.5	488 nm	~482	~695	Far-red
PerCP-eFluor710	PerCP-eFluor710	488 nm	~482	~710	Far-red

Fluorophore class/family	**Common fluorophores**	**Typical excitation laser**	**Ex. max (nm)**	**Em. max (nm)**	**Typical spectral region**
Allophycocyanins	APC	633/635/640 nm	~650	~660	Far-red
APC-Cy7	APC-Cy7	633/640 nm	~650	~780	Near-IR
APC-eFluor780	APC-eFluor780	633/640 nm	~650	~780	Near-IR
Alexa Fluor red/far-red	AF594	561/594 nm	590	617	Red
	AF647	633/640 nm	650	668–671	Far-red
	AF700	633/640 nm	702	~723	Far-red
	AF750	633/640 nm	752	776	Near-IR
Brilliant Violet (BV)	BV421	405 nm	~416	~421	Violet-blue
	BV510	405 nm	~405	~510	Green
	BV570	405 nm	~405	~570	Yellow
	BV605	405 nm	~405	~605	Orange
	BV650	405 nm	~405	~650	Red
	BV711	405 nm	~405	~711	Far-red
	BV786	405 nm	~405	~786	Near-IR
Brilliant Ultraviolet (BUV)	BUV395	UV (~355 nm)	~348	~395	Violet
	BUV496	UV (~355 nm)	~348	~496	Blue-green
	BUV737	UV (~355 nm)	~348	~737	Far-red
Pacific Blue family	Pacific Blue	405 nm	~410	~455	Blue

Fluorophore class/family	**Common fluorophores**	**Typical excitation laser**	**Ex. max (nm)**	**Em. max (nm)**	**Typical spectral region**
Pacific Orange	Pacific Orange	405 nm	~405	~551	Orange
eFluor dyes	eFluor 450	405 nm	~405	~450	Blue
	eFluor 506	405 nm	~405	~506	Green
	eFluor 605	405 nm	~405	~605	Orange
	eFluor 660	633/640 nm	~633	~660	Far-red
Cyanine dyes	Cy3	532/561 nm	~550	~570	Orange
	Cy5	633/640 nm	~650	~670	Far-red
	Cy5.5	633/640 nm	~675	~694	Near-IR
	Cy7	633/640 nm	~750	~775	Near-IR
DNA-binding dyes	DAPI	UV/405 nm	~358	~461	Blue
	7-AAD	488 nm	~546	~647	Red
	Propidium iodide (PI)	488 nm	~535	~617	Red
Cell-permeable dyes	CFSE	488 nm	~492	~517	Green
Fluorescent proteins	GFP	488 nm	~488	~507	Green
	YFP	488/514 nm	~514	~527	Yellow
	mCherry	561 nm	~587	~610	Red
	CFP	405 nm	~433	~475	Cyan

2. Fluorescence Compensation

Because fluorochromes often emit overlapping signals, compensation is essential.

The guidelines recommend:

- Using single-stained controls.
- Calculating compensation for every experiment.
- Avoiding manual adjustments without validation.
- Verifying compensation before data collection.

Practical importance: Prevents false-positive and false-negative signals.

3. Standard Gating Strategy during FACS analysis

Accurate gating is fundamental to reliable flow cytometry analysis. Gating strategy should be logical, reproducible, transparent and fully documented.

The recommended workflow includes:

1. Remove debris.
2. Exclude doublets.
3. Exclude dead cells.
4. Identify leukocytes.
5. Sequentially identify immune cell subsets.

Following standard gating strategy, data analysis and statistics brings out outstanding FACS result.

It is further recommended to present the experiment with:

- Transparent reporting of methods.
- Documentation of software versions.
- Validation of analytical workflows.

It is important to note that proper analysis ensures meaningful and reproducible conclusions.

4. Guidelines for Rare-Cell Detection

- Define the expected frequency of the target population before acquisition.
- Acquire sufficient total events to obtain statistically meaningful numbers of rare cells.
- Use Poisson statistics when determining the required number of events.
- Consider pre-enrichment when the target population is extremely rare.

- Select markers that provide unambiguous identification.
- Use appropriate FMO and biological controls.
- Optimize sample concentration and flow rate.
- Consider dump channels to remove unwanted populations.
- Report both the number of target events and total events acquired.

5. Reporting Standards

To improve reproducibility, it is suggested to report:

- Antibody clones and fluorochromes used in the experiment.
- Instrument model and configuration.
- Sample preparation methods.
- Gating strategy.
- Compensation procedures.
- Quality control measures.
- Statistical analyses.

Complete reporting enables other laboratories to reproduce and validate findings.

6. Quality Assurance

The guidelines advocate continuous quality management through:

- Regular instrument maintenance.
- Staff training.
- Standard operating procedures (SOPs).
- Internal and external quality assessments.
- Validation of new reagents and protocols.

Quality assurance minimizes variability and improves long-term reliability.

Clinical and Translational Applications

Standardized flow cytometry protocols support research into:

- Autoimmune diseases
- Cancer immunotherapy
- Infectious diseases
- Vaccine development

  FACS enable researchers to:

  - Measure antigen-specific T cells
  - Assess memory responses
  - Evaluate cytokine production
  - Monitor vaccine efficacy
- Organ transplantation
- Primary immunodeficiencies
- Hematological disorders

Reliable immune profiling helps clinicians and researchers better understand disease mechanisms and evaluate therapeutic responses.

Specialized Applications of FACS

Modern FACS platforms support increasingly complex applications in cell biology, immunology, and translational research, including:

- High-dimensional and spectral flow cytometry for multiparameter cellular profiling
- Cell-cycle and proliferation analysis
- Rare-cell detection and characterization
- Immune monitoring in cancer and autoimmune diseases
- Infectious disease research, including SARS-CoV-2 studies
- Vaccine-response evaluation
- Stem-cell characterization
- Minimal residual disease (MRD) assessment
- Hematological disorder investigation

Functional Flow Cytometry application in Immunology

In immunology, FACS can be efficiently used to evaluate immune cell functions rather than just phenotype.

Some of the main applications of FACS in immunology include:

- Cytokine production
- Intracellular staining
- Cell proliferation
- Cell activation
- Cytotoxicity
- Apoptosis
- Calcium signalling
- Oxidative stress

These assays help researchers understand how immune cells respond under different conditions.

FACS Applications Across Multiple Fields

The real strength of these guidelines lies in their broad practical applicability.

Academic Research

Researchers benefit by higher quality publications from the impressive results outcomes from the FACS experiments with improved reproducibility.

Clinical Laboratories

Clinical diagnostic laboratories can improve immune monitoring, Leukemia immunophenotyping, minimal residual disease detection and Biomarker validation.

Pharmaceutical Research

Drug developers can optimally design FACS experiments during immunotherapy development, clinical trials, safety testing and pharmacodynamic monitoring

Precision Medicine

As personalized medicine continues to expand, reliable immune profiling becomes increasingly important. Standardized flow cytometry supports individualized treatment decisions by enabling accurate characterization of patient immune responses.

Reference:

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