BECKMAN COULTER
Life Sciences

Unveiling the Hidden Signals: Overcoming Autofluorescence in Spectral Flow Cytometry Analysis

What is Autofluorescence?

Autofluorescence (AF) refers to the inherent fluorescence exhibited by cells due to the presence of certain cellular components and metabolites that fluoresce upon excitation by specific wavelengths of light.[1] Common endogenous fluorophores include cyclic compounds such as reduced pyridine nucleotides (NADH), oxidized flavin coenzymes (FMN and FAD), vitamins, and proteins containing aromatic amino acids like tryptophan, phenylalanine and tyrosine.

Additionally, in tissues, structural proteins in the extracellular matrix (ECM), such as collagen and elastin, can contribute to AF. Certain cells also contain lipopigments such as lipofuscin that are a result of lipid oxidation and contribute to cellular AF.[2] Plant and algal cells also possess inherent autofluorescent compounds. Plants exhibit AF due to chlorophyll, flavonoids, and structural components like lignin, suberin, and sporopollenin.[3] Similarly, algal cells display intrinsic AF due to pigments involved in photosynthesis such as chlorophylls, phycobiliproteins and carotenoids.[4] **Figure 1** depicts the emission wavelengths of common autofluorescent molecules in different cell types, highlighting their diverse emission spectra.

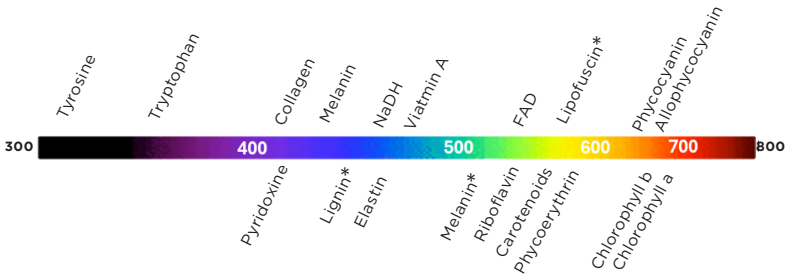

Figure 1: Emission wavelengths range of common autofluorescent molecules across various cell types (humans, animals, plants) from 300-800 nm. (★) Lignin has two distinct emission wavelengths depending on excitation wavelength: one around 400 nm and another around 500-550 nm. Emission wavelengths for pigments like melanin and lipofuscin have a broad range from (450-650 nm) and (460-630 nm) respectively.

These natural fluorophores serve as intrinsic markers, offering insights into cellular processes, metabolic activities, and physiological conditions without a need for external dyes or labels. Consequently, AF serves as a valuable tool for monitoring cellular dynamics. Changes in cells and tissues in connection with the onset of any metabolic condition modify the amount and distribution of fluorophores. For instance, increased AF has emerged as an established marker for cellular senescence and aging across various cell types, including human mesenchymal stromal cells, nerve cells and skin cells. Such changes in AF levels are also observed in cancer cells.

Challenges Arising from Autofluorescence in Flow Cytometry

AF serves as a valuable indicator of cellular processes and states, aiding in the identification of cell populations and assessment of cell health and function in conventional flow cytometry. However, it can also pose challenges by potentially masking specific fluorescent markers, particularly those emitting weaker signals, leading to inaccurate measurement. As illustrated in **Figure 1**, various compounds in human and animal cells naturally emit light in the wavelength range of 400-600 nm. AF leads to reduced signal resolution and sensitivity in this spectral region, notably affecting commonly used dyes like FITC or Alexa Fluor 488.

The intensity of AF correlates directly with cell size and granularity. Larger cells such as megakaryocytes and macrophages, which contain higher levels of autofluorescent molecules, exhibit elevated AF. Moreover, AF is dynamic and can fluctuate based on factors such as the metabolic state of the cell. For example, dead cells, which accumulate autofluorescent compounds, typically exhibit high AF levels. Certain cell types inherently display heightened AF due to specific cellular components. For instance, myeloid cells contain granules rich in autofluorescent compounds, while antigen-presenting cells possess a high mitochondrial content, contributing to their natural AF. As shown in **Figure 2** detection of elevated autofluorescence (AF) levels in unstained plant extracts suggests potential complications for subsequent experimental analyses. Experimental procedures and staining techniques, such as aldehyde fixation and nutrient availability, also influence cellular AF levels, adding to the complexity and variability of AF in cellular analysis.

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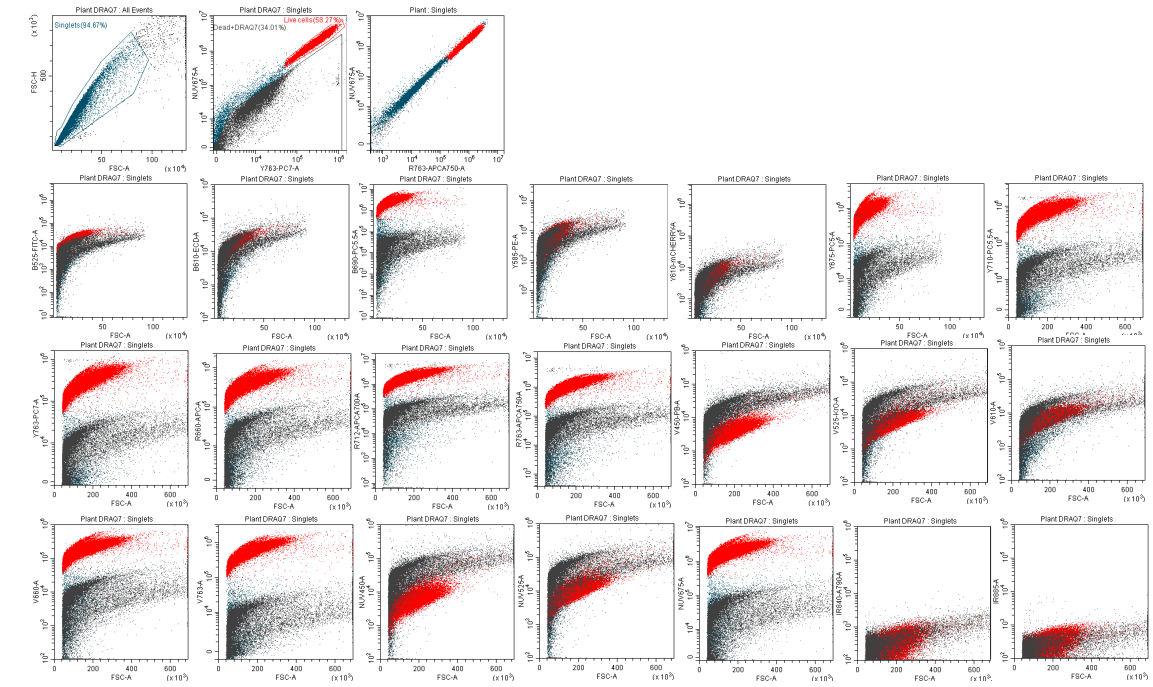

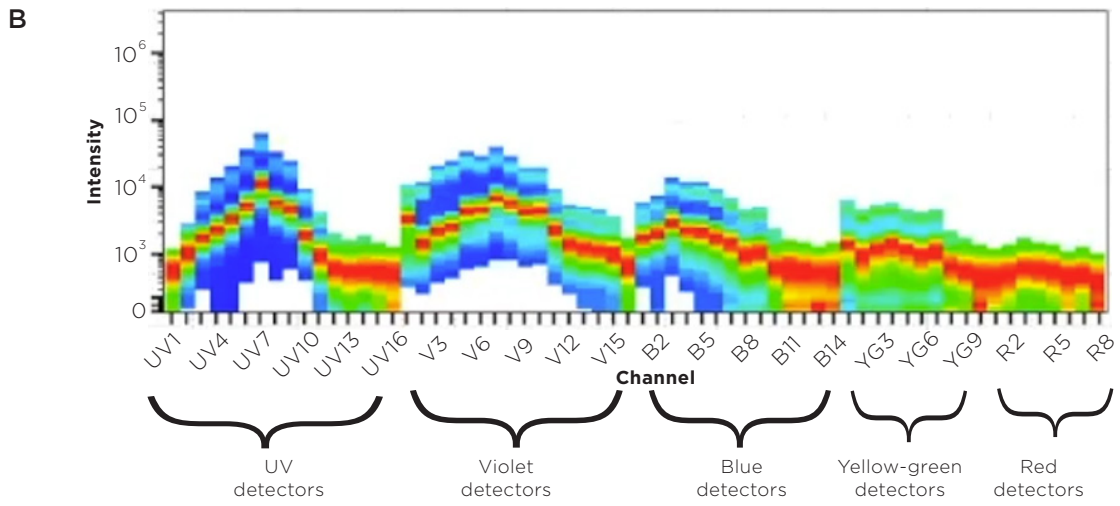

Figure 2: Presence of differential autofluorescence in cells. **A.** Flow Cytometric Analysis of Plant Cells: A flow cytometric dot plot illustrates the autofluorescence (AF) observed in plant cells, revealing differential AF across various channels attributable to distinct cellular components and pigments. Notably, the highest fluorescence is detected in specific channels, which can be attributed to the emission properties of autofluorescent plant pigments such as chlorophyll. The plant extract was prepared following Galbraith's method,[5] with the buffer replaced by PBS, and the analysis was conducted using the CytoFLEX LX flow cytometer. (Data shared by Dr. Alfonso Blanco, Director of the Flow Cytometry Core Technologies at University College Dublin (UCD)) **B.** Spectral signature of unstained, fixed whole blood sample seen using a spectral flow cytometer.

AF poses a significant challenge in flow cytometry, impacting the precision of marker detection and quantification across diverse research fields. In cancer research, it hinders the identification of specific, low-abundance markers crucial for diagnostic and therapeutic advancements. Similarly, in immunology, autofluorescent compounds such as lipofuscin obscure the accurate characterization of immune cell subsets, complicating studies on immune responses and diseases. Metabolically active stem cells also exhibit heightened AF, which can obscure the detection of critical markers essential for understanding stem cell dynamics and differentiation pathways. In microbiology, the intrinsic fluorescence of certain bacterial and fungal species complicates microbial identification and quantification, affecting research on microbial ecology and pathogenesis. In plant biology, AF originating from chlorophyll and flavonoids interferes with investigations into cellular structures, photosynthesis mechanisms, and plant responses to environmental cues.

Addressing these complexities is crucial for refining fluorescence-based methodologies, enhancing measurement accuracy, and ensuring the reliability of findings. Efforts to mitigate AF effects involve advanced analytical techniques, novel fluorophores with distinct emission spectra, and optimized experimental protocols tailored to specific research objectives. Because traditional flow cytometry does not allow researchers to extract AF, spectral flow cytometry can be used to extract AF during unmixing.

Autofluorescence Hurdles in Spectral Flow Cytometry

Spectral flow cytometry is a cutting-edge technology that significantly enhances the capabilities of conventional flow cytometry by capturing the entire emission spectrum of each fluorophore across a broad range of wavelengths. This is achieved by employing an array of detectors that collect continuous spectral data for each fluorescent tag, generating a unique spectral signature for it. This distinctive signature is subsequently used to unmix and identify the specific spectrum of each fluorescent molecule from a multicolor sample. However, the analysis of spectral flow cytometry data can be complicated by the presence of cells' intrinsic AF, which can contribute significantly to background. This autofluorescent background can interfere with the precise detection of the desired fluorescence signals, particularly when studying low-abundance markers or when trying to identify dim populations **(Figure 3)**. As a result, it can be challenging to distinguish these cells from the background, leading to potential underestimation of these populations.

The presence of high AF reduces the sensitivity of detecting weakly fluorescent markers, making it difficult to accurately identify positive and negative populations.[6] In such cases, if AF is not properly extracted, the positive and negative populations may appear almost indistinguishable. Therefore, it is essential to develop and implement effective strategies to mitigate the impact of AF in spectral flow cytometry analysis to ensure accurate and reliable results.

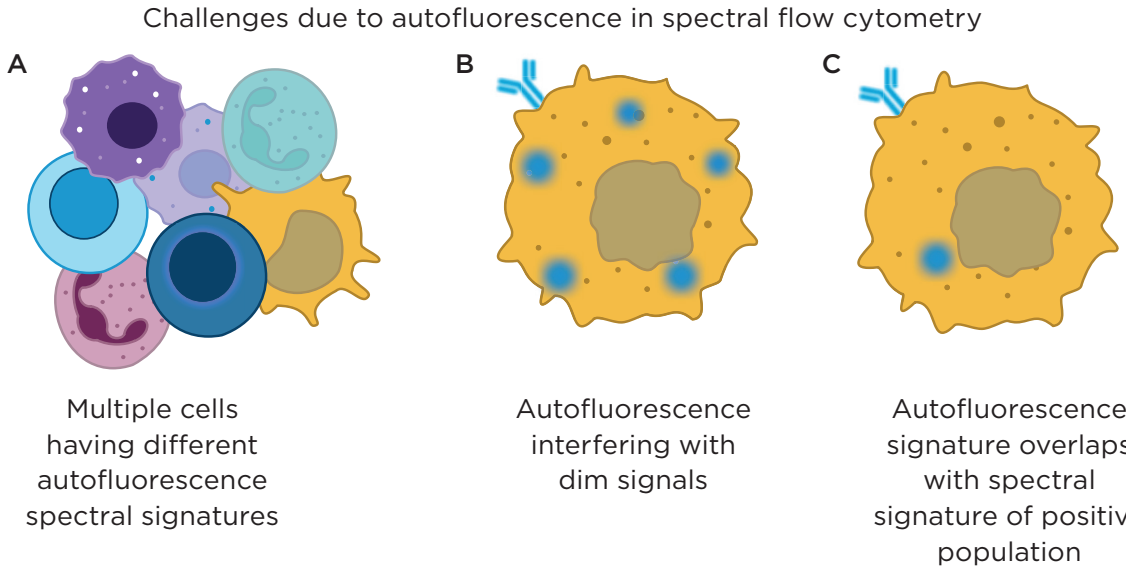

Figure 3: Illustrating common challenges due to autofluorescence in spectral flow cytometry. **A.** Heterogeneous autofluorescence profiles across different cell types, resulting from varying levels of endogenous fluorophores and cellular components leading to multiple autofluorescent signals. **B.** Difficulty in distinguishing dim populations from background due to high cellular autofluorescence. **C.** Overlapping spectral signatures of desired fluorescent tag and autofluorescence, leading to signal interference.

Beyond the Noise: Strategies for Autofluorescence Management in Spectral Flow Cytometry

AF in certain cases can be used to characterize cells, but in some cases, AF significantly impacts the accuracy of flow cytometry results. One strategy to mitigate this issue involves selecting channels that do not overlap with autofluorescent emissions or using fluorophores that are brighter than the cellular AF. Another approach entails lowering the detector voltages to minimize the visual interference caused by AF. This method, however, also reduces the detector's sensitivity to the desired fluorochrome, which may compromise analytical precision. These approaches can still be used in conventional bandpass flow cytometry, but they are not feasible in the context of spectral flow cytometry.

- **In simple samples**: Each autofluorescent unstained cell has a unique spectral signature. The distinct AF characteristics of the sample can be identified and used to develop new panels by treating the spectral signature of AF as an additional endogenous dye and applying effective panel design. This can help in distinguishing the unique spectral contributions of AF, which can then be computationally removed from the signals of interest. After extracting autofluorescent signals, users can accurately analyze fluorescence from specific markers or targets. AF extraction is crucial in spectral flow cytometry for identifying and characterizing low-abundance markers, unmasking dim signals, and delineating the expression and subset distribution of low-abundance markers, thereby enhancing the resolution of positive populations.[7]

- **In samples with heterogeneous AF**: In spectral cytometry, accurate characterization of AF signatures is crucial for effective unmixing of stained cells and achieving reliable results. However, the application of a single average spectrum to a mixture of cells with diverse autofluorescent signatures can lead to unmixing failures. This issue is particularly pronounced when samples comprise multiple cell types exhibiting varying levels of AF.

Moreover, even within a single sample, cells may display heterogeneous autofluorescent profiles, stemming from their unique metabolic state, size, composition, or environmental influences, rendering it difficult to implement a universal compensation or unmixing strategy. This heterogeneity can be visualized by a high AF intensity in raw, unstained sample, and it complicates spectral flow cytometry, as each autofluorescent cell or sample may produce a distinct spectral signature. In such cases, use of diverse unstained controls is crucial, as it enables researchers to effectively account for the inherent variability in autofluorescent intensities exhibited by different cell types.

In recent years, a few interesting studies[7,8,9] have been published describing methods to discover multiple AF signals from samples using spectral flow cytometry, indicating growing interest in this field. Strategies include manually gating distinguishable populations using channels that show high variation in AF intensity, or employing unmixing techniques with AF extraction to visualize AF with each fluorochrome- and gate-distinguishable population. Additionally, dimension reduction algorithms followed by metaclustering can identify a greater number of gated populations by focusing on clusters with sufficient event counts. As the total AF is an aggregate of multiple cell types, by analyzing the autofluorescent profiles from various unstained control samples, researchers can discern cell populations exhibiting elevated levels of intrinsic AF. Upon identification, the most accurate number and combination of AF signatures can be incorporated into the unmixing matrix. Users can check the results from unmixing later to identify if AF has been correctly unmixed from sample autofluorescence.

- **Samples that get spectrally altered upon binding target**: Additionally, a few fluorophores such as tandem polymer dyes are susceptible to spectral alterations depending on their binding target. Typically in these cases, verification of either a bead-based or cell-based control yields the optimal unmixing outcome. This differential analysis enables the identification and subsequent gating out of autofluorescent signals, thereby minimizing their impact on the detection and analysis of positive fluorescent populations.

Conclusion

AF presents significant challenges in flow cytometry, particularly in spectral flow cytometry, complicating the detection, quantification and interpretation of fluorescence signals. Heterogeneous autofluorescence, varying across cell types and components, further complicates analysis. Addressing these challenges requires a comprehensive understanding of the sample, meticulous experimental design, rigorous unmixing techniques, and appropriate controls to enhance data accuracy and reliability. Using multiple channels to differentiate autofluorescent signals from specific fluorophores is crucial, and spectral flow cytometers capable of handling multiple autofluorescent signals simultaneously can help in this respect. Additionally, advanced spectral unmixing algorithms and appropriate compensation controls can better isolate true signals from noise, significantly improving the precision and reliability of flow cytometry experiments.

References

1. Monici, M. 2005. "Cell and Tissue Autofluorescence Research and Diagnostic Applications." Biotechnology Annual Review 11: 227-256.

2. Katz, M. L., and W. G. Robison Jr. 2002. "What Is Lipofuscin? Defining Characteristics and Differentiation from Other Autofluorescent Lysosomal Storage Bodies." Archives of Gerontology and Geriatrics 34, no. 3 (May-June): 169-184.

3. Donaldson, L. 2020. "Autofluorescence in Plants." Molecules 25, no. 10: 2393.

4. Takahashi, T. 2019. "Routine Management of Microalgae Using Autofluorescence from Chlorophyll." Molecules 24: 4441.

5. Galbraith D., Villalobos-Menuey E. Analysis of Plant Genome Sizes Using Flow Cytometry: A Case Study Demonstrating Dynamic Range and Measurement Linearity. Available online: https://www.mybeckman.ru/resources/reading-material/application-notes/plant-genome-size-flow-cytometry-analysis

6. Lokwani, R., R. Chaudhari, et al. 2022. "Spectral Cytometry on Highly Autofluorescent Samples." Nature Reviews Methods Primers 2: 71.

7. Jameson, V. J., et al. 2022. "Unlocking Autofluorescence in the Era of Full Spectrum Analysis: Implications for Immunophenotype Discovery Projects." Cytometry Part A 101, no. 11: 922-941.

8. Kharraz, Y., et al. 2022. "Full Spectrum Cytometry Improves the Resolution of Highly Autofluorescent Biological Samples: Identification of Myeloid Cells in Regenerating Skeletal Muscles." Cytometry Part A 101, no. 10: 862–876.

9. Peixoto, M. M., et al. 2022. "Identification of Fetal Liver Stroma in Spectral Cytometry Using the Parameter Autofluorescence." Cytometry Part A 101, no. 11: 960–969.

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2024-GBL-EN-106675-v1