Unmixing the [Fluorescent] Spectrum of Sperm Cellular Health Utilizing High-Parameter, Image-based, Spectral Flow Cytometry

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Sperm cell health evaluation is crucial for understanding potential male fertility status. Flow cytometry (FC) has been utilized to observe sperm characteristics since the 1970s; however, a limiting function of traditional flow cytometry is that fluorescence overlaps between fluorophores used can crowd one another and limit the number of biomarkers analyzed per assay. Recent advancements have enabled full fluorescent spectrum analysis to observe the unique fluorescent fingerprints of fluorophores. This advancement, called spectral FC, is achieved due to by having a high number of detectors (e.g. >25) compared to more traditional (e.g. <15) FC along with computational unmixing of the fluorescent spectrum. Previously, the maximum number of fluorophores used in sperm quality analysis was reported to be five. We hypothesize that the use of spectral FC will enable the use of a high number (>10) fluorophores. The BD FACS Discover™ S8, equipped with 78 detectors and image and sorting capability, allows for high parameter subpopulation integration and analysis. Specifically, for sperm, this means the combination of high-throughput and high-parameter cell health and morphology quantification which was previously impossible. In this study, we aimed to multiplex the application of 19 fluorophores for sperm biomarker health determination. Sperm capacitation status; viability; plasma membrane integrity & potential; membrane fluidity; acrosomal membrane integrity; zinc signature; intracellular pH; intracellular calcium; cellular amines; mitochondrial mass, function, activity, and potential; reactive oxygen species (ROS) production (Nitric oxide and total ROS); lipid peroxidation; caspase activity; and cell death due to apoptosis were all targeted simultaneously in this assay. For this purpose, the following fluorophores were used, PNA-PE, LCA-Cy5, Sytox™ Red, $DiSBAC_2(3)$, Merocyanine 540, FluoZin™-3-AM, BCECF-AM, Rhod-5N-AM, Zombie Green, MitoTracker™ Green FM, MitoTracker™ Red CMXRos, MitoTracker™ Deep Red FM, JC-1, 4,5-diaminofluorescein diacetate (DAF-2 DA), CellROX™ Orange, BODIPY™ 581/591 C11, CellEvent™ Caspase-3/7 Green, and PE/Dazzle™ 594 Annexin V. Additionally, Hoechst 33342 was used as a nuclear dye for detection of single cellular events and exclude debris from downstream analysis. Following single stain sample acquisition, FlowJo™ (v10.10) software was used to calculate a spectral similarity matrix and spectral unmixing. All of the fluorophores used were able to be successfully spectrally unmixed except for Zombie™ Green, MitoTracker™ Green, and DAF-2 DA. These three fluorophores were too similar (similarity index ≥ 0.99), meaning two of the three fluorophores should be excluded. These results indicate that up to 17 of the 19 fluorophores in the present study can be used in combination to increase subpopulation segregation of sperm for detailed cellular health analysis. Results here support increased sperm quality parameters for the evaluation of fertility potential in livestock sires and men. This project was supported by Agriculture and Food Research Initiative Competitive Grant No. 2022-67015-36298 from the USDA National Institute of Food and Agriculture.