Evaluation of interacting cells in peripheral blood by flow cytometry: an understudied area

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Abstract

Flow cytometry traditionally focuses on immunophenotyping of single cells. Doublets, multiplets, coincident cells, and debris are typically removed from analysis. Biologically, cells of many types interact transiently or for longer periods. Whether such cell interactions may be important in distinguishing pathological conditions, and what the reference ranges are in healthy individuals, is an understudied area. In this abstract, we first developed methods using BD CellView™ Imaging Technology on the BD FACSDiscover™ A8 Cell Analyzer to identify interacting cells, such that they are resolved distinctly from cells that happen to be coincident but non-interacting. Combining imaging derived parameters such as delta center of mass and radial moment were particularly useful in this regard. We then piloted the approach on PBMCs from healthy donors. In data across 15 healthy donors, T-cell x B-cell interactions averaged ~0.70% of CD3 T-cell singlets (CV 35%), T-cell x monocyte ~0.90% (CV~75%), and T-cell x NK-cell ~0.38% (CV~50%). Compared to singlet populations, the T-cell x monocyte doublets were strongly enriched in CD8 T-cells, while the T-cell x B-cell and T-cell x NK-cell interactions were modestly enriched in CD4 T-cells. The methods developed in this study can now be applied to deepen understanding of the roles of various immune cell phenotypes within synaptic doublets and how their frequencies may vary across health and disease.

Methods

PBMCs from 15 healthy donor blood samples were isolated using SepMate tubes with a Ficoll density gradient. Cells were counted and assessed for viability. 6 million cells were used for each donor for the fully stained panel to assess frequency of complexes and 1 million cells were used for each single-color control, except for CD34 which used BD SpectraComp™ Beads.

PBMCs were stained with cocktailed antibodies in the presence of BD Brilliant Stain Buffer Plus for 30 minutes at room temperature in the dark. Following incubation, cells were washed twice with wash buffer (2% FBS in PBS). After the final wash, cells were re-suspended Cell d in stain buffer and acquired on a BD FACSDiscover™ A8 Cell Analyzer using BD FACSChorus™ Software. 500,000 doublets were recorded with images for each donor. Interacting cells were assessed using a combination of fluorescent and image derived gates.

The reagent panel is shown below. Markers with blue laser excitation were directly imaged, including CD3, CD14, CD19. Additional markers could be used to infer identity of cells involved in doublets confirmed by label free imaging.

13 Color Interacting Cell Panel

Marker	Fluorochrome	Clone	Marker	Fluorochrome	Clone
Brilliant Stain Buffer Plus			CD56	BV786	NCAM16.2
CD45	BUV395	HI30	CD25	RV828	2A3
CD16	BUV496	3G8	CD3	BB515	UCHT1
HLA-DR	BUV615	G46-6	CD14	RB613	MφP9
CD34	BV421	581	CD19	RB705	SJ25C1
CD8	V500	RPA-T8	CD45RA	APC	HI100
CD45	BV711	SK3	CD20	APC-Cy7	L27

Imaging Channels

BD FACSDiscover™ A8 Cell Analyzer



- 13 color panel (3 color imaging)
- 15 donors screened
- Default pre-optimized settings

Platform	BD FACSDiscover™ A8 Cell Analyzer (spectral + real-time imaging)
Technologies	BD SpectralFX™ (system-aware unmixing); BD CellView™ Image Technology (camera-free)
Lasers	UV 349 nm/30 mW, V 405/50, B 488/100, YG 561/50, R 637/100; 3L, 4L or 5L configurations
Spectral detectors	78 APDs: UV 22, V 20, B 16, YG 12, R 8 (365–860 nm)
Scatter	Blue FSC, SSC, ALL; Violet SSC, ALL
Imaging	3 blue-excited FL channels (534/46, 600/60, 788/225) + FSC/SSC/ALL; 78 imaging parameters from 12 morphometric features
Acquisition	35,000 events/s spectral; 12,500 events/s imaging
Sample handling	Integrated autoloader (96-well plates, 40-tube racks), 4/20/37°C
Software	BD FACSChorus™; FCS 3.2 export; FlowJo™ with BD CellView™ Lens plugin

Imaging by BD CellView™ Technology in BD FACSDiscover™ A8 uses frequency encoded light to image at typical flow cytometry speeds

Results (1): Image gating verifies synaptic doublets

Figure 1:

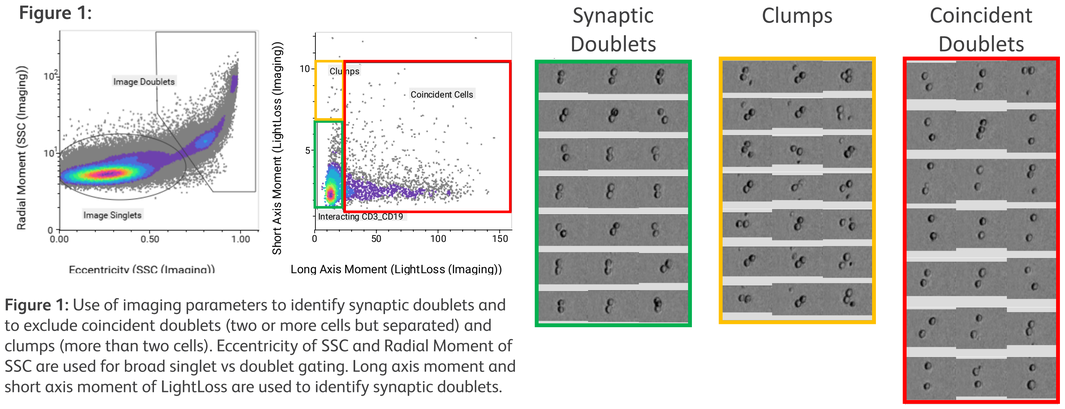


Figure 1: Use of imaging parameters to identify synaptic doublets and to exclude coincident doublets (two or more cells but separated) and clumps (more than two cells). Eccentricity of SSC and Radial Moment of SSC are used for broad singlet vs doublet gating. Long axis moment and short axis moment of LightLoss are used to identify synaptic doublets.

Results (2): Synaptic doublets are robust and can be sorted

Figure 2:

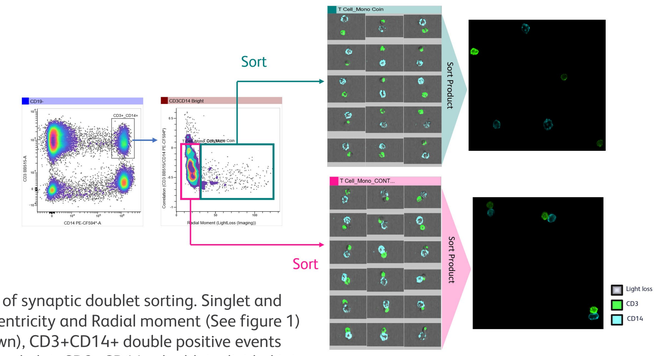


Figure 2: Demonstration of synaptic doublet sorting. Singlet and doublet gating using Eccentricity and Radial moment (See figure 1) were used (plots not shown), CD3+CD14+ double positive events were gated on in the second plot. CD3+CD14+ doublets divided into synaptic (pink gate) and coincident (blue gate) and both populations were sorted. Coincident sort products distribute much as singlets would- random T cells and monocytes spread over the slide, while synaptic doublets sort as pairs and remain engaged. Note: Synaptic doublet survival is donor dependent, and some interactions are stronger than others.

Results (3): Fluorescence and image gating methodology to identify synaptic doublets involving T, B, and NK cells

Figure 3:

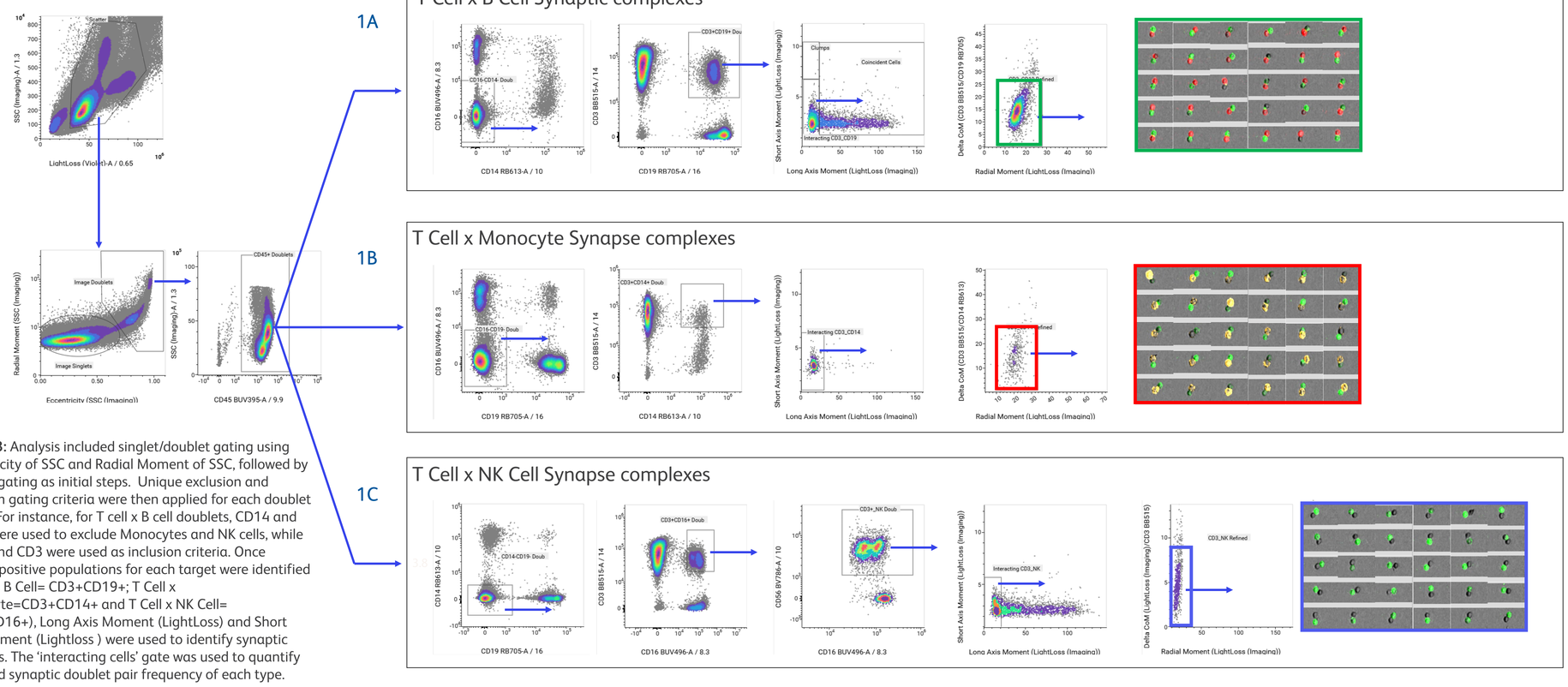
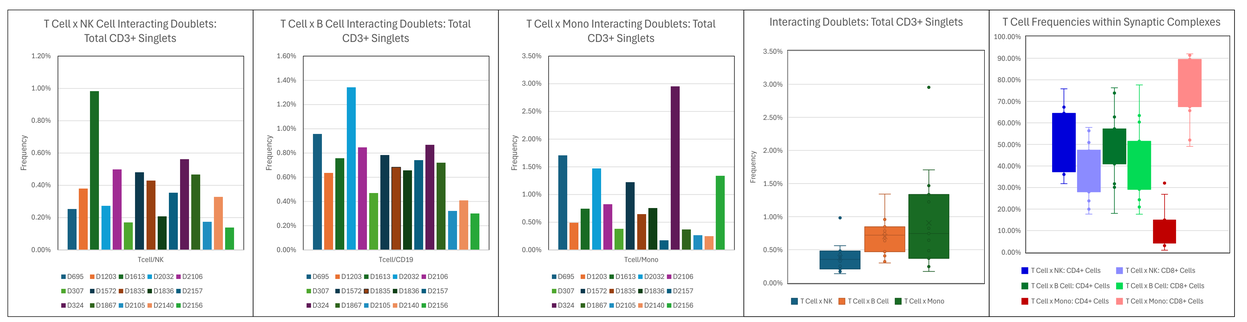


Figure 3: Analysis included singlet/doublet gating using Eccentricity of SSC and Radial Moment of SSC, followed by CD45+ gating as initial steps. Unique exclusion and inclusion gating criteria were then applied for each doublet target. For instance, for T cell x B cell doublets, CD14 and CD16 were used to exclude Monocytes and NK cells, while CD19 and CD3 were used as inclusion criteria. Once Double positive populations for each target were identified (T Cell x B Cell= CD3+CD19+; T Cell x Monocyte=CD3+CD14+ and T Cell x NK Cell= CD3+CD16+), Long Axis Moment (LightLoss) and Short Axis moment (Lightloss) were used to identify synaptic doublets. The 'interacting cells' gate was used to quantify engaged synaptic doublet pair frequency of each type.

Results (4): Frequencies of synaptic doublets across 15 heathy donors

Figure 4:



Conclusions

- Image gating was developed to distinguish synaptic doublets from larger clumps and coincident cells
- Synaptic doublets identified by BD CellView™ Imaging Technology were robust as confirmed by sorting and microscopy
- Combining image and fluorescent gating enabled analysis of various interacting cells including T-cell x B-cell, T-cell x monocyte, and T-cell x NK-cell.
- Initial ranges were explored across 15 healthy donors, indicating average frequencies of doublets compared to singlet cells
  - ~0.70% T-cell x B cell (CV~35%)
  - ~0.90% T-cell x monocyte (CV~75%)
  - ~0.38% T-cell x NK-cell (CV ~50%)
- This work opens the door to further phenotyping of doublets and investigation of doublet frequencies in health and disease

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