

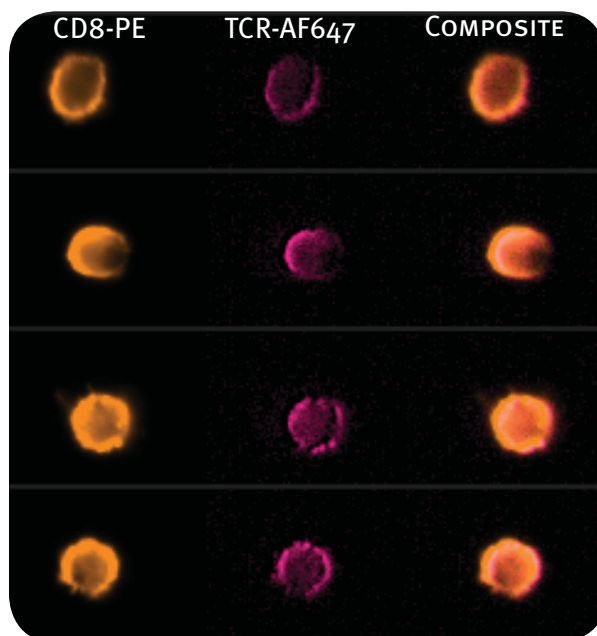
FRET analysis of protein-protein interaction and redox sensor folding using the ImageStream

IMAGESTREAM CAPABILITIES FEATURED: Internalization / Cell Signaling & Molecular Translocation / Cell-Cell interaction / Morphology / Cell Cycle & Mitosis / **Co-localization** / Spot Counting / DNA Damage & Repair / **Cell Death & Autophagy** / Immunology / **Biochemistry** / Oncology / Virology / Microbiology / Parasitology / Hematology / Stem Cell Biology / Oceanography / Toxicology / Drug Discovery

ABSTRACT

FRET (Förster or Fluorescence Resonance Energy Transfer) occurs when an excited donor fluorophore transfers energy to an acceptor fluorophore, resulting in reduced donor fluorescence and enhanced acceptor fluorescence. FRET occurs only when the donor/acceptor pair are in close proximity (1-10 nm), and thus is a useful technique to measure direct protein-protein interactions and conformational changes. While FRET can be quantified using conventional flow cytometry, microscopy is often required to confirm interpretation of results. Here we show FRET measurement with different fluorophore combinations and experimental applications using the ImageStream imaging cytometer, which enables quantitative FRET measurement directly from images of a large numbers of cells per sample.

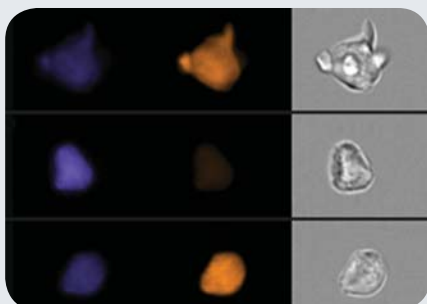
The ImageStream's unique ability to measure the location of the FRET signal on, within, or between cells even in rare subpopulations should prove invaluable for understanding signaling pathways in the future.



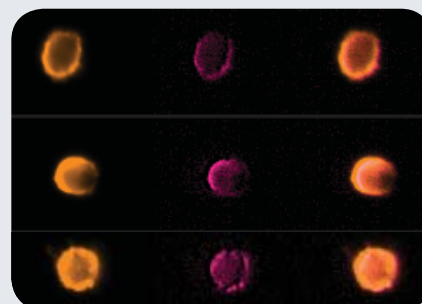
Anti-CD8-PE interaction with anti-TCR-AF647

Study Highlights

Oxidizing conditions induce conformational change in redox-sensing peptide, resulting in CFP:YFP FRET.



CD8-TCR co-localization following stimulation is determined by measuring PE (donor) anti-TCR to AF647 (acceptor) anti-CD8 FRET.

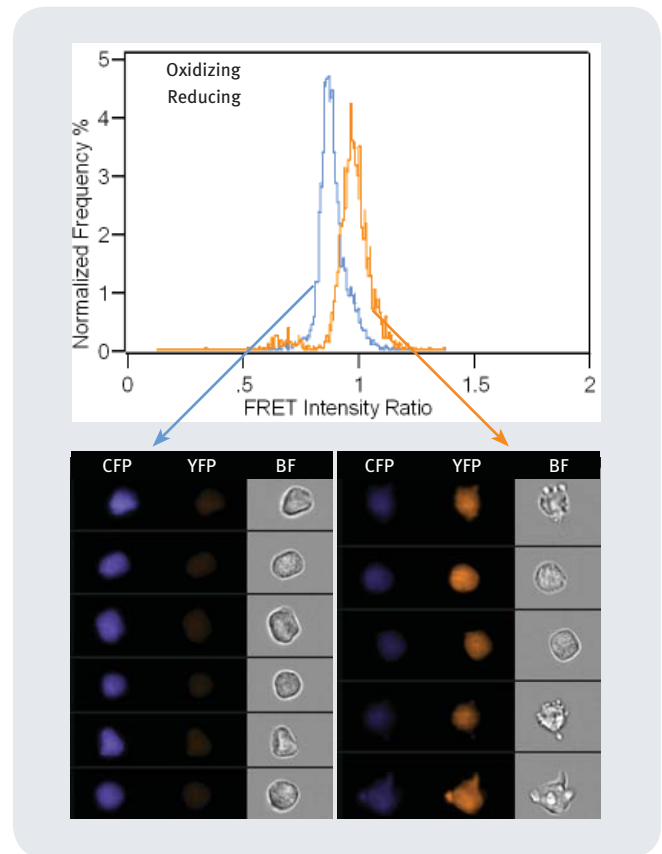
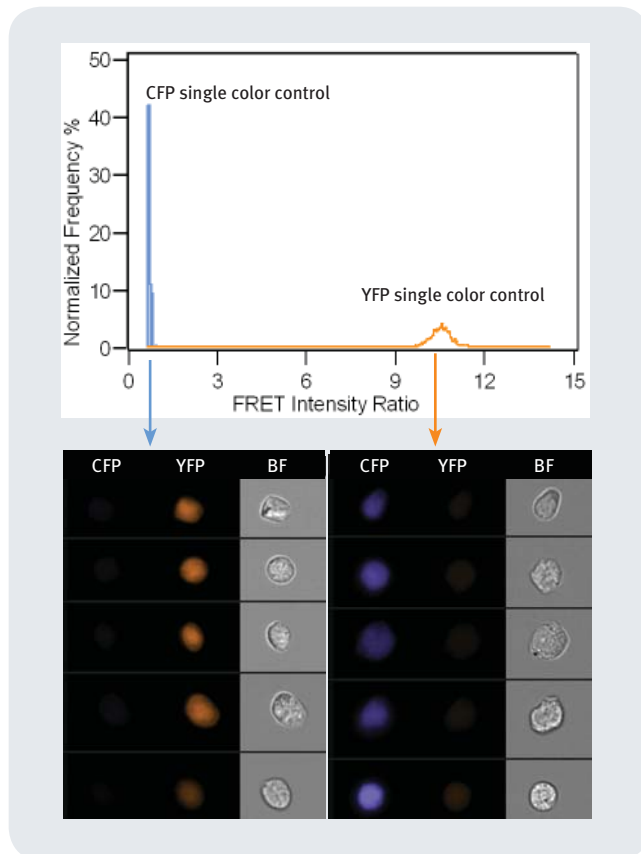


CELLULAR REDOX STATE REPORTED BY CFP:YFP FRET OF CHIMERIC PEPTIDE

Vladimir Kolosov, University of Illinois Urbana-Champaign

Cellular redox status is determined using a CFP/YFP dual labeled reporter peptide. Oxidizing conditions induce a conformational change in the reporter, bringing CFP and YFP in close enough proximity for CFP-YFP FRET to occur. FRET was measured from

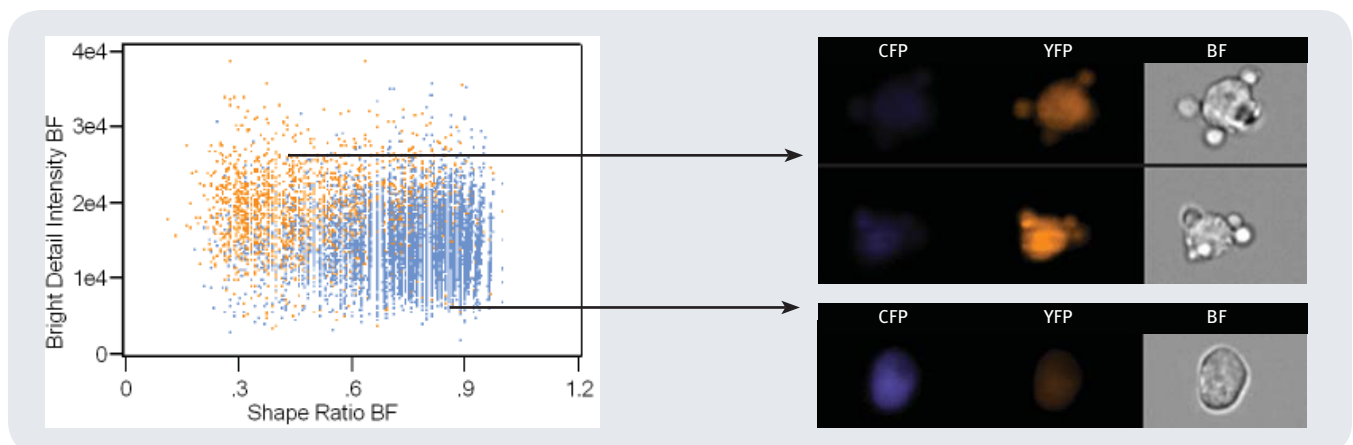
images obtained by 405 nm laser excitation on the ImageStream using the FRET (YFP:CFP) Intensity Ratio feature. The plot at right shows that cells exposed to oxidizing conditions (orange) have higher FRET Ratio values compared to control cells (blue).



MORPHOLOGY DIFFERENCES ACCOMPANYING REDOX CHANGE

Cells exposed to oxidizing conditions showed increased texture and blebbing, morphologic hallmarks of apoptosis. The plot of brightfield Shape Ratio (surface

uniformity) vs Bright Detail Intensity (blebs appear as bright spots) indicates that oxidative conditions (orange) induce apoptosis.



CD8-TCR INTERACTION VIA PE:AF647 - IMAGESTREAM COMPARED WITH FLOW CYTOMETRY

Gregoire Wieers, Ludwig Institute

CD8-TCR association within a T cell line was determined following stimulation for 5 or 14 days by measuring anti-CD8-PE (donor) to anti-TCR-AF647 (acceptor) FRET. One method for measuring the amount of fluorescence transferred from donor to acceptor commonly employed in conventional flow cytometry is calculation of the “FRET unit” (Doucey, M.A., *et al.*, JBC, 278, 3257) – see formula below. The FRET Units obtained by ImageStream analysis compared well to conventional flow analysis (see table).

$$\text{FRET Unit} = (E_{31,1} - E_{30,0}) - ([E_{30,1} - E_{30,0}] \times [E_{21,1}/E_{20,1}]) - ([E_{31,0} - E_{30,0}] \times [E_{11,1}/E_{11,0}])$$

Where E₁ is PE fluorescence measured with 488 nm excitation, E₂ is AF647 fluorescence from 658 nm excitation, and E₃ is AF647 fluorescence from 488 nm excitation of PE with subsequent FRET.

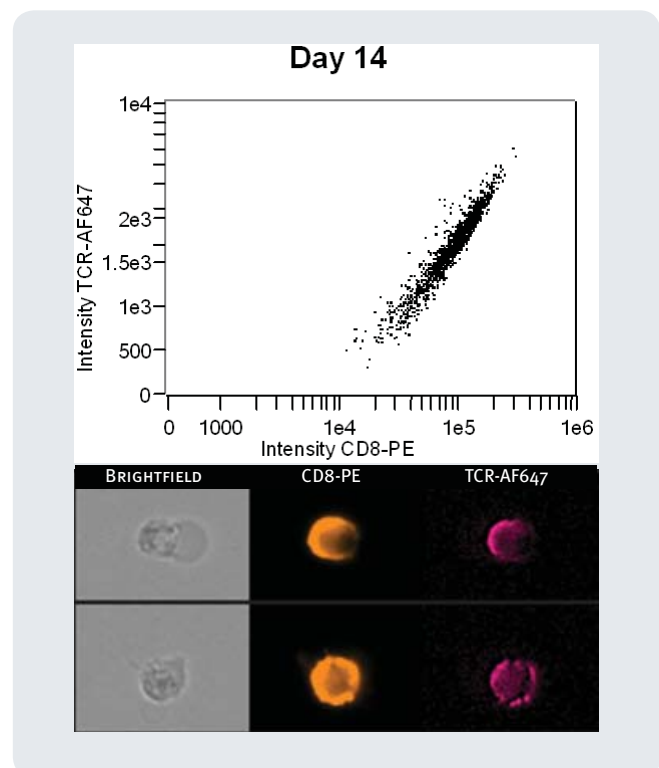
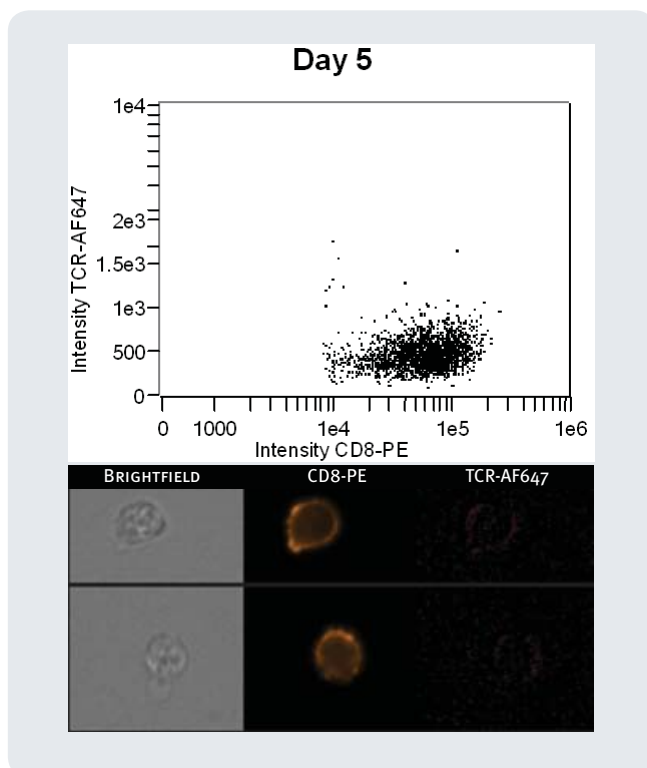
- 1,1 = sample labeled with both fluorophores
- 1,0 = sample labeled with PE only
- 0,1 = sample labeled with AF647 only
- 0,0 = unlabeled sample

	D5 ImageStream	D14 ImageStream	D5 Flow Cyt.	D14 Flow Cyt.
	CD8 PE TCR AF647	CD8 PE TCR AF647	CD8 PE TCR AF647	CD8 PE TCR AF647
E1 0,0	4.19	4.32	2.37	3.34
E2 0,0	1.00	1.00	2.53	2.96
E3 0,0	2.35	2.02	2.24	3.29
E1 1,0	644.90	853.74	407.00	602.00
E2 1,0	1.00	1.00	2.35	3.18
E3 1,0	60.40	78.70	74.10	115.00
E1 0,1	5.56	4.17	2.11	2.38
E2 0,1	103.35	152.26	241.00	473.00
E3 0,1	2.74	2.42	2.63	3.95
E1 1,1	575.55	986.51	393.00	639.00
E2 1,1	97.08	147.80	229.00	478.00
E3 1,1	55.18	108.96	72.20	134.00
FRET Units	0.66	1795	0.20	11.47

CD8-TCR INTERACTION VIA PE:AF647 - ANALYSIS USING COMPENSATED DATA

While molecular co-association can be quantified with FRET units, microscopy is often required to confirm interpretation of results. The plots below show the day 5 and day 14 post-stimulation dual labeled samples with

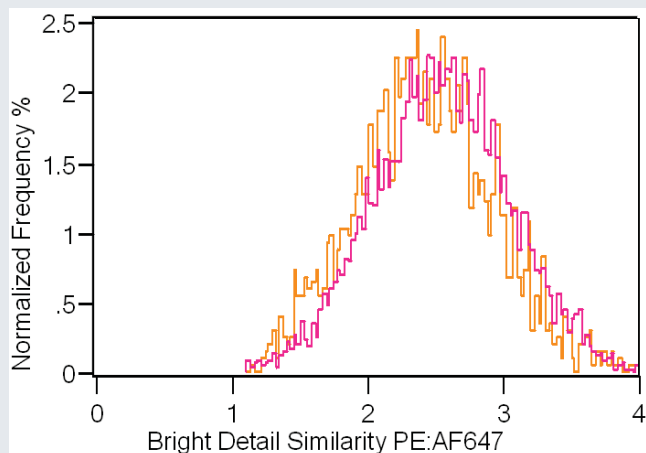
488 nm excitation only. These plots are generated from compensated data, so any detectable red fluorescence comes from AF647 and not spectral crosstalk from PE. Thus CD8 and TCR have become associated by day 14.



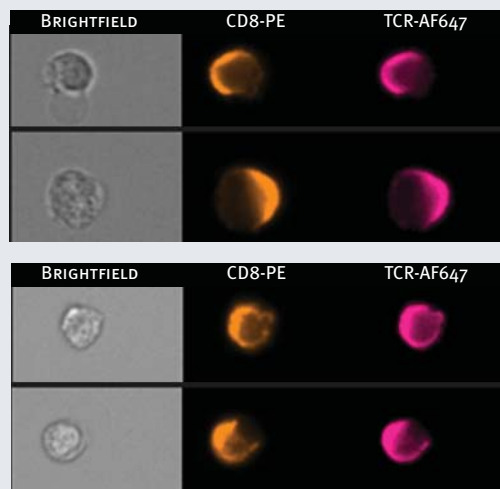
MEASUREMENT OF CO-LOCALIZATION USING BRIGHT DETAIL SIMILARITY

Proteins may co-localize to a functional supra-molecular complex without directly interacting with one another. We measured the extent of CD8/TCR co-localization (488/658 nm excitation) in the day 5 and day 14 samples using the Bright Detail Similarity feature. This feature correlates the bright regions of the CD8/TCR image pair for each cell. Those cells with co-localized molecules will have CD8 and TCR images that look alike, and consequently will have high

values. As shown in the plots below, cells from both time points have high CD8/TCR Similarity values, consistent with the images shown on the left (day 5 on top, day 14 on bottom). In conjunction with the FRET data previously described, these data indicate that while CD8 and TCR are co-localized at both time points, they become directly associated (FRET positive) with one another by day 14.



Orange = Day 5 and Red = Day 14 after stimulation



ImageStream^x Specifications



EXCITATION SOURCES

LASER (NM)	EXAMPLE DYES
405	DAPI, Pacific Blue™
488	FITC, PE, ECD, PE-Cy5
560	Alexa Fluor® 546, Cy3
592	Texas Red®, Alexa Fluor® 594
658	Cy5, Alexa Fluor® 647, APC, APC-Cy-7

INSTRUMENT CAPABILITIES

Images per Cell	Up to 12
Imaging Modes	Brightfield, SSC, and fluorescent
Sample Throughput	1 sample/min nominal
Automated Processes	Startup, shutdown, and self-calibration

Pacific Blue™, Alexa Fluor®, and Texas Red® are trademarks of Life Technologies Corporation. Cy® is a trademark of GE Healthcare. ECD® is a trademark of Beckman Coulter, Inc. DRAQ5™ is a trademark of Biostatus, Ltd.

IMAGING PERFORMANCE

Magnification	20X	40X	60X
Numeric Aperture	0.5	0.75	0.9
Field of View (µm)	120 x 1024	60 x 512	40 x 340
Imaging Rate (cells/sec)	2,000	1,000	600