

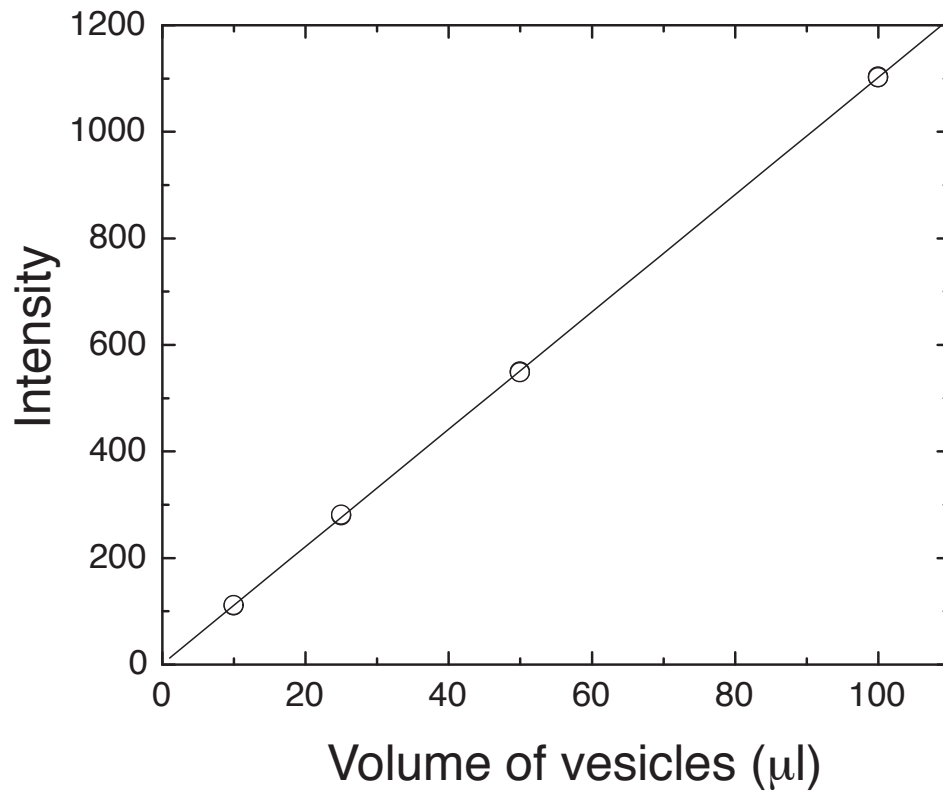
Supplementary Figure 1: *Scattering from vesicles is negligible.* Fluorescence intensity of various concentrations of the same BODIPY-DHPE containing vesicles diluted into 1 ml buffer, measured on the microscope as described in the Materials and Methods section, and fit to a linear function. The observed linearity indicates that scattering is not significant across this concentration regime. All observations of vesicle fluorescence used for calculating correction factors lie within this range.

Supplementary Figure 2: *Components of $\Phi(\lambda_{ex}, \lambda_{em})$.* (a) Optical components that affect the excitation of a fluorophore on our microscope setup are the Hg lamp, dichroic mirror reflectance, and excitation filter, whose normalized spectra are shown. (b) Fluorophore emission is affected by the emission filter, dichroic mirror transmission, and camera sensitivity, with spectra as indicated. The combination of the three spectra shown in (a) (or (b)) is shown on the excitation (or emission) axis of (c). The two vectors representing these products are multiplied to create the $m \times n$ matrix $\Phi(\lambda_{ex}, \lambda_{em})$ graphed in (c). Even within the relatively constant transmission regions of the filters, the sharp features of the lamp emission can serve to create large differences in brightness between fluorophores with similar spectra. The Hg lamp spectrum was provided by Sylvania for an HBO 103W/2 lamp, the dichroic and filter spectra (Chroma filter set HQ TR #41004) were downloaded from www.chroma.com, and camera sensitivity data for the Photometrics Coolsnap HQ were downloaded from www.photomet.com. Spectra were reinterpolated as needed using OriginPro (Origin Labs) to have the appropriate step size between wavelengths (1.5 nm for excitation, 2 nm for emission).

Supplementary Table 1: Numerical values of F

	F measured on the microscope	F measured by fluorimetry
AlexaFluor568 anti-biotin	0.80 ± 0.03	0.70 ± 0.08
AlexaFluor568 anti-TCR F_{ab}	0.9 ± 0.1	0.89 ± 0.02
AlexaFluor594 anti-biotin	0.82 ± 0.03	0.81 ± 0.03
AlexaFluor594 ephrin	0.59 ± 0.06	0.565 ± 0.006
DsRed monomer	0.1311 ± 0.0009	0.119 ± 0.002
Texas Red avidin	0.11 ± 0.01	0.128 ± 0.009
Texas Red neutravidin	0.158 ± 0.009	0.167 ± 0.007
Texas Red streptavidin	0.124 ± 0.005	0.121 ± 0.006
AlexaFluor488 anti-biotin	1.3 ± 0.1	1.24 ± 0.07
AlexaFluor488 streptavidin	0.39 ± 0.03	0.49 ± 0.03
BODIPY anti-IgG	0.71 ± 0.05	0.71 ± 0.05
eGFP	0.807 ± 0.004	0.6886 ± 0.0008
eYFP	0.760 ± 0.004	0.6523 ± 0.0007
eYFP-ICAM	0.81 ± 0.08	0.70 ± 0.04
FITC anti-biotin	0.88 ± 0.08	0.88 ± 0.06
Oregon Green anti-biotin	0.60 ± 0.05	0.54 ± 0.03

Supplementary Figure 1



Supplementary Figure 2

