# Two-photon Fluorescence Resonance Energy Transfer Stoichiometry in Living Cells

**Amar R. Bhagwat[1], Daniel Flynn[2], Meredith H. Brenner[3], Marcos Nunez[4], Jennifer P. Ogilvie[1,4], Dawen Cai[5] and Joel A. Swanson[6]**

[1]*Dept. of Physics, University of Michigan, 450 Church St., Ann Arbor, MI 48109, USA*
[2]*Dept. of Macromolecular Science and Engineering, University of Michigan, 2300 Hayward Ave., Ann Arbor, MI 48109, USA*
[3]*Applied Physics Program, University of Michigan, 450 Church St., Ann Arbor, MI 48109, USA*
[4]*Dept. of Biophysics, University of Michigan, 930 N. University Ave., Ann Arbor, MI 48109, USA*
[5]*Dept. of Cell and Developmental Biology, University of Michigan Medical School, 109 Zina Pitcher Place., Ann Arbor, MI 48109, USA*
[6]*Dept. of Microbiology and Immunology, University of Michigan Medical School, 1150 W. Medical Center Dr., Ann Arbor, MI 48109, USA*
*amarb@umich.edu*

**Abstract:** Using phase-shaped pulses, we perform, for the first time, a proof-of-principle demonstration of two-photon fluorescence resonance energy transfer (FRET) microscopy for studying the stoichiometry of intermolecular interactions within living cells.
**OCIS codes:** (320.5540) Pulse shaping; (190.4180) Multiphoton processes; (180.4315) Nonlinear microscopy

Fluorescence-resonance energy transfer (FRET) is a short-range non-radiative transfer of excitation from one fluorophore (donor, D) to another (acceptor, A) [1]. Even though the process is non-radiative, the excitation transfer requires that there is significant overlap between the emission spectrum of the donor with the excitation spectrum of the acceptor. FRET efficiency is proportional to $R^{-6}$, where R is the inter-fluorophore distance and is significant for values of $R < 10$ nm. Because of this strong distance dependence, FRET has been used as a spectroscopic ruler for studying intermolecular interactions and biological processes inside living cells [2,3]. By tagging two interacting molecules with fluorophores from a FRET pair, the interaction can be heralded by the occurrence of FRET. In addition, FRET has also been used to study the stoichiometry of such an interaction [4] so that the relative concentrations of the two molecules and the ratio in complex can be deduced.

Stoichiometry studies that use one-photon interactions require the use of either a mercury lamp equipped with filter wheels (slow switching between illumination conditions)[4] or multiple narrow bandwidth lasers (difficult alignment and registering of images). Using pulse-shaping, a single broad-bandwidth pulsed laser can afford fast, selective excitation of the donor or acceptor, while eliminating the need to overlap multiple laser sources [5]. It also permits the use of two-photon excitation; such interactions have reduced photo-damage, increased penetration depth, and intrinsic z-sectioning capability [6]. In this paper, we extend the one-photon experiment performed by Hoppe *et al.* [4] to the two-photon case and in the process derive a generalized two-photon theory for FRET stoichiometry to account for the resulting additional interactions.

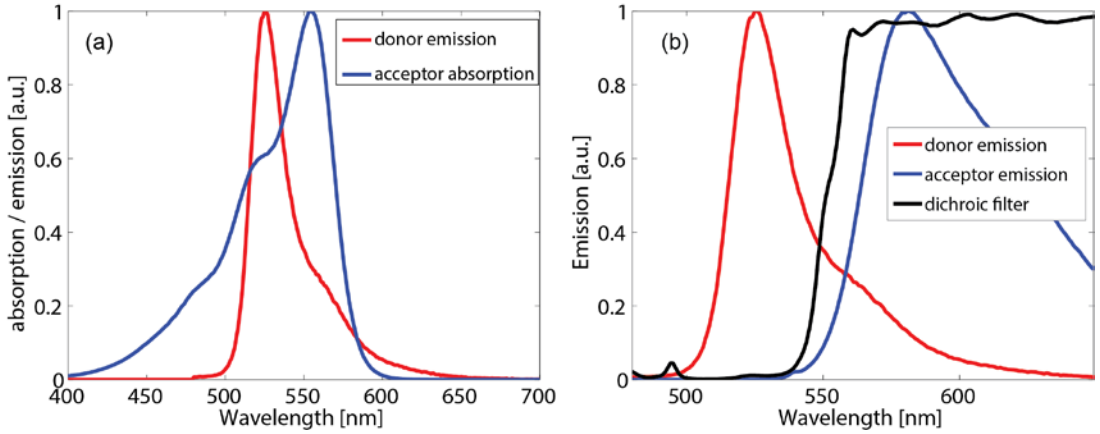


Fig. 1: (a) Overlap of donor (mAmetrine) emission and acceptor (tdTomato) absorption spectrum. (b) Donor and acceptor emission spectra superimposed with transmission profile of the dichroic filter that separates the collected fluorescence into the two detection channels

Samples for imaging are obtained by transfecting Cos-7 cells with plasmids encoding fluorescent proteins that form a FRET pair (in our case, mAmetrine (Am) and tdTomato (TdT)). Figure 1(a) shows the spectral overlap between the donor emission (Am, red line) and acceptor absorption (TdT, blue line), while Fig. 1(b) shows the corresponding emission spectra of the fluorophores as well as the transmission of the dichroic used for splitting the

fluorescence into the two detection channels labeled donor and acceptor emission. Selective excitation of the fluorophores is performed by spectral-phase-shaping the excitation pulses such that various fundamental laser frequencies constructively interfere only over a part of the two-photon spectrum. The required spectral phase is calculated using a genetic algorithm and applied using a binary phase-shaping technique [7].

The experimental setup consists of a broadband Ti:Sapphire (Ti:S) laser (650 – 1050 nm, Venteon Pulse:One), phase-shaped with an SLM-based pulse-shaper (Biophotonic Solutions MIIPS box 640P) to selectively excite the acceptor or donor (in addition to dispersion compensation) and routed to an upright microscope (Olympus BX51WI) via an X-Y scan head (Prairie Tech.). The cells are maintained at 37ºC during observation using a heated stage in Ringer's buffer and imaged with a water-immersion objective (Olympus UPlanApo 60X, 1.2 N.A.). The epi-fluorescence is split with a dichroic (595 DCXR) into two PMT channels (donor and acceptor emission), pre-amplified and digitized with a fast ADC synchronized with the scan head to properly register the images. Additionally, the FRET efficiency used in the data analysis is measured using a commercial fluorescence lifetime setup (FLIM).

The significant overlap of both the excitation as well as emission spectra of donor and acceptor (Fig. 1) requires us to calculate overlap constants using cells expressing only Am or only TdT. Additionally, a linked only Am-TdT cells are used for calculating system absorption cross-sections and quantum yield ratios. Finally, cells expressing both Am and TdT with only a fraction of the fluorophores linked as a FRET pair act as a test sample for quantifying the FRET stoichiometry. Following a procedure analogous to [3], we use the above constants and three imaging conditions ($D_{exc}$-$D_{em}$, $D_{exc}$-$A_{em}$, and $A_{exc}$-$A_{em}$) to obtain fraction of acceptor in complex ($f_A$), fraction of donor in complex ($f_D$), and the ratio of acceptor to donor (R).

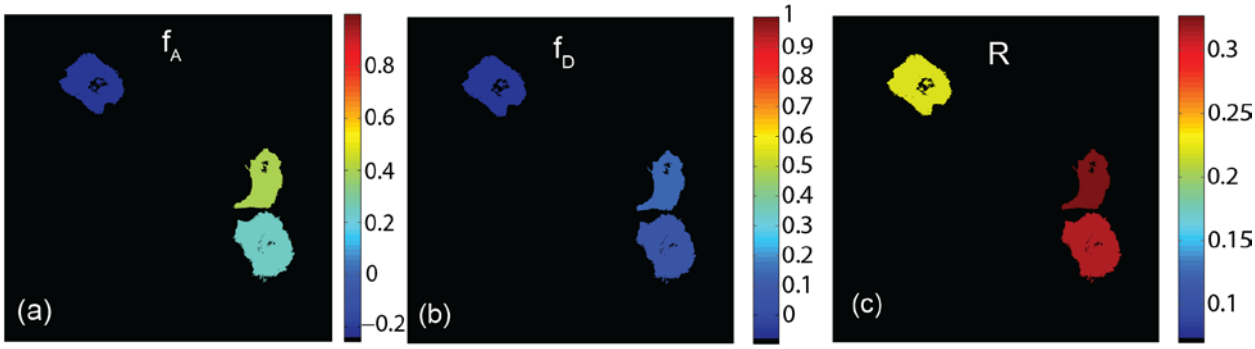


Fig. 2: Images of cells showing (a) ratio of acceptor in complex to total donor, (b) ratio of donor in complex to total acceptor, (c) ratio of total acceptor to donor in the prepared cells.

In our samples, the linked FRET pair is separated by a fixed 8-amino acid linker, and FLIM data gathered separately for free donor and only FRET pair indicates the FRET efficiency of our linked construct to be ~30%. Figure 2 shows preliminary analysis for a collection of cells where excess donor and acceptor were expressed along with the linked construct. We see that for all cells, we observe the ratios $f_D$, $f_A$ and R to be within a reasonable range and that the stoichiometry can be calculated. We are currently in the process of gathering data on an increased sample size as well as other FRET pairs to build statistics for measuring the accuracy and robustness of the method.

In conclusion, we use phase-shaped pulses to perform selective two-photon excitation of the donor or acceptor in a FRET pair and show a proof-of-principle demonstration of two-photon FRET stoichiometry of intermolecular interactions within living cells for the first time.

## References

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