

the different receptors expressed by these cells which are used for antigen internalization and processing. We are comparing antigen presentation mediated through CD64 (the Fc receptor pathway), CD86/CD80 (the B7-1/B7-2 pathway), and CD40, all of which load peptides (antigens) into the MHC Class II compartment, for ultimate delivery back to the cell surface. That is the process which presents foreign antigens to various lymphocytes in order to initiate immune responses. With live cell, multicolor confocal microscopy, we are attempting to determine the extent of redundancy and inter-regulation among these pathways. For example, when, if ever, do molecules entering recycling endocytic pathways via these three different receptor pathways merge? How are the signaling events associated with the individual pathways related spatially and temporally to the endocytosed antigens and carriers? And, do the signals generated by these receptors modulate each others' function, either positively or negatively? Of interest and concern is the task of separating opportunity from artifact in both collecting and analyzing such complex data sets.

## Reference

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## The Greatly Reduced Photodamage of 2-Photon Microscopy Enables Extended Three-Dimensional Time-Lapse Imaging of Living Neurons

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Confocal microscopy has provided a breakthrough in resolution for fluorescently labeled thick specimens, providing depth discrimination not possible when using wide-field fluorescence microscopy. However, the intensity of illumination necessary to produce a satisfactory image using confocal laser-scanning microscopy (CLSM) is often sufficient to photobleach the fluorophores used and to damage living specimens (phototoxicity). These problems are multiplied when a z series of images is taken to produce a three-dimensional (3-D) reconstruction of the specimen due to the increased light dose. For this reason, it has not been possible to image more than a few z sections of a living specimen for an extended period without bleaching the label and/or killing the specimen.

Two-photon laser-scanning microscopy (TPLSM) provides all of the benefits of CLSM, with greatly reduced photodamage (Denk *et al.* 1990, 1995). This has allowed, for the first time, nondestructive time-lapse fluorescence imaging

of living neurons, in a substantial volume of tissue, across an extended time period. This was accomplished using a 2-photon microscope, built with minor changes to a Molecular Dynamics Sarastro 2000 CLSM that allows excitation from a pulsed infrared (IR) titanium:sapphire laser. Unlike with CLSM, with TPLSM there is no excitation of the dye above or below the focal plane. Only within a diffraction-limited spot at the focus is the intensity sufficiently large to achieve 2-photon excitation. This greatly reduces the total light dose and eliminates the need for a confocal aperture. With pulsed illumination at 850–900 nm, we imaged cultured neurons labeled with a number of popular green- or blue-excitable labels, including DiI, DiA, DiO, Bodipy ceramide, and Green Fluorescent Protein (Potter and Fraser 1995).

We are using cultured slices of rat hippocampus as a model system to test silicon “neuroprobes” designed to provide a long-term, two-way connection between neural tissue and external electronics. The neuroprobes have wells designed to hold the soma of a neuron in close proximity to a stimulation/recording electrode. We label dissociated embryonic rat hippocampal neurons with DiO before placing them into the neuroprobe wells to allow observation of the process of neurite outgrowth and synaptic integration with the host slice using TPLSM. To determine whether cultured slices from postnatal-Day 9 rats are permissive to the growth of the probe neurons, we seeded dissociated, DiO-labeled embryonic-Day 18 hippocampal neurons directly onto cultured slices. The labeled “transplanted” cells were grown for one day on the slice and then imaged using 3-D TPLSM time lapse. One day after seeding, the transplants had migrated throughout the thickness of the slice and extended into many neurites, some several hundred  $\mu\text{m}$  long. We imaged a volume of  $250 \times 250 \times 40 \mu\text{m}$ , containing over 20 labeled cells, every 15 min for over 7 h. No evidence of photobleaching or phototoxicity was observed; the neurons were as bright and active at the end of the imaging session as at the beginning. Numerous fine neurites and growth cones were visible without image enhancement. Several somata continued to migrate, and neurite retractions were almost as common as extensions. These experiments demonstrate the usefulness of TPLSM in studying the morphologic dynamics of living neurons in three dimensions over extended time periods.

## References

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