Imaging Agents

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Genetically Encoded pH Sensor for Tracking Surface Proteins through Endocytosis**

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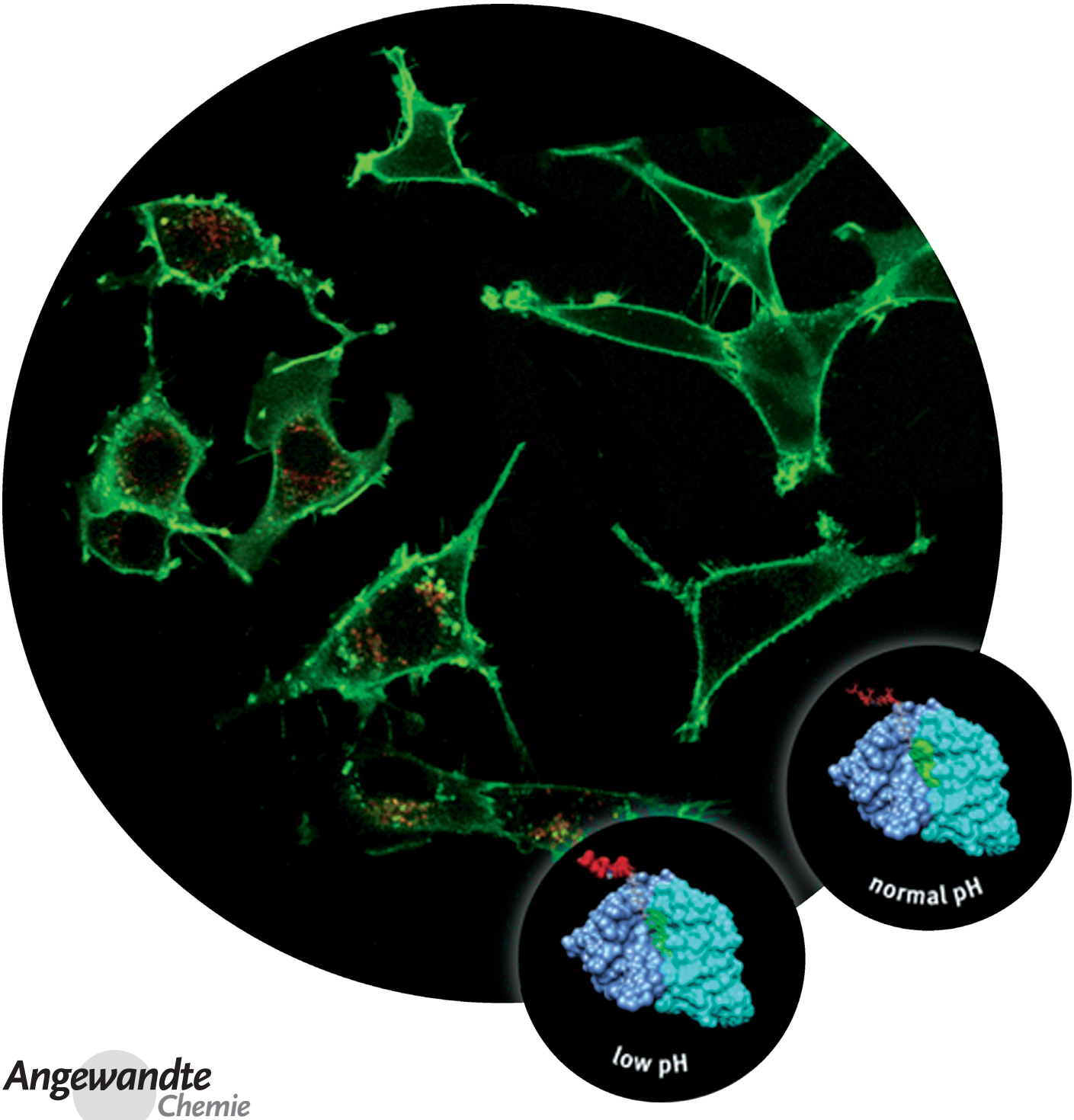

We have combined our fluorogen-activating peptide[1] with a new tandem dye molecule to develop a biosensor that labels a cell-surface protein and displays an easily detectable pH-dependent color change in emission through efficient intramolecular Förster resonant energy transfer. This probe has demonstrated pH variations in β_2-adrenergic receptor trafficking and revealed a process of surface-to-endosome intercellular transfer in dendritic cells having potential significance in antigen transfer.

The fate and localization of proteins through the endocytic pathway is of interest across a broad range of biological investigations because inappropriate trafficking of surface proteins can lead to various diseases.[2–4] Bulk methods for pH measurement where pits are loaded with two dyes, one pH dependent (such as fluorescein) and the other pH independent (such as rhodamine), provide the aggregate endolysosomal pH rather than trafficking-related changes associated with a single protein.[5] Genetically tagged fluorescent proteins (pHluorins) exhibit activation (ecliptic) or excitation ratio (ratiometric) signatures in response to pH changes.[6] However, these reporters are not selective for surface proteins, thus requiring image-based segmentation to select the subset of cell surface proteins. Typical "surface selection" is accomplished using total internal reflection fluorescence (TIRF) that images up to about 100 nm in depth, which is far greater than the 5–10 nm thickness of the plasma membrane.[7] This, however, does not truly select for events occurring at the surface of the cell, nor does it allow tracking of these events further into the cellular volume than the TIRF field.

For true cell-surface-selective imaging, antibodies are conjugated to the pH-dependent CypHer5 dye.[8] The antibodies bind to cell surface proteins that are accessible without permeabilization (i.e., not in collared pits).[9] The fluorescence increase at low pH values allows determination of the relative acidity of the internalized vesicles, but determination of the precise pH value is difficult.[10] Typical ratiometric dyes such as the seminaphthorhodofluors (SNARFs) are not available as conjugates.[11] Ligands coupled to ratiometric DNA nanosensors were recently used to track ligand-associated pH changes through the endocytic pathway.[12] Despite these advances, imaging of the cell surface subset of receptor proteins and their fate in live cells remains a considerable challenge.

We have previously demonstrated that exposing an expressed fluorogen-activating peptide (FAP)[1,13] to a cell-impermeant, fluorogenic dye, labeled the subset of proteins present at the cell surface. This approach chemically discriminates between the proteins at the plasma membrane and those within secretory or endocytic compartments, and allows selective imaging of the transport of these proteins. To convert this label into a physiological indicator, we have coupled a pH-dependent Cy5 analogue (II) to a fluorogen donor, derived from thiazole orange (TO1), at a distance to allow efficient Förster resonant energy transfer (FRET). This provides a cell-excluded reagent that is activated upon binding to an expressed tag only when it is exposed at the plasma membrane. The bound dye displays a pH-dependent dual-band emission spectrum, a result of the protonation of the Cy5 derivative II, thus producing increased spectral overlap and FRET efficiency at low pH values.[14]

Figure 1a illustrates the tandem dye and the pH-dependent emission spectra of the HL1.0.1-TO1 FAP tandem dye complex in citrate/phosphate buffer from pH 4.0 to pH 8.0 (Figure 1b). A systematic variation in spectral overlap and Förster radius between the fluorogen TO1 (thiazole orange derivative) and the pH-dependent Cy5 derivative (II) leads to a significant change in the ratio of red to green emission (see Table S1, and Figures S1 and S2 in the Supporting Information). The FAP/tandem dye complex gives the same ratiometric trend both in vitro and on the mammalian cell surface, thereby demonstrating a standardizable readout of this pH biosensor (Figure 1c, and Figure S3). The dye shows significant fluorogenic activation (ca. 10-fold) in response to binding to the FAP. The tandem dye binds to its cognate FAP with a $K_d = 55$ nM and with a fluorescence quantum yield of 0.06 (at pH 4). The pK_a value of the probe determined by the fluorescence ratio is 6.4 ± 0.05.

Such a reagent in effect combines the properties of the ecliptic and ratiometric pHluorins, that is, chemoselective labeling of cell surface proteins which allows subsequent measurement of pH changes in response to trafficking of the labeled molecules (Scheme 1). This type of measurement is not yet available for studies in living cells. The β_2-adrenergic receptor (β_2AR) is a canonical G-protein coupled receptor with well-characterized trafficking. Stimulation of the receptor with an agonist results in rapid clathrin-mediated endocytosis with subsequent actin-dependent recycling to the plasma membrane.[15–18] This receptor is an important catecholamine receptor that is expressed in the brain, heart, lung, and a number of other tissues, and is a therapeutic target for asthma and cardiovascular diseases. Retroviral trans-

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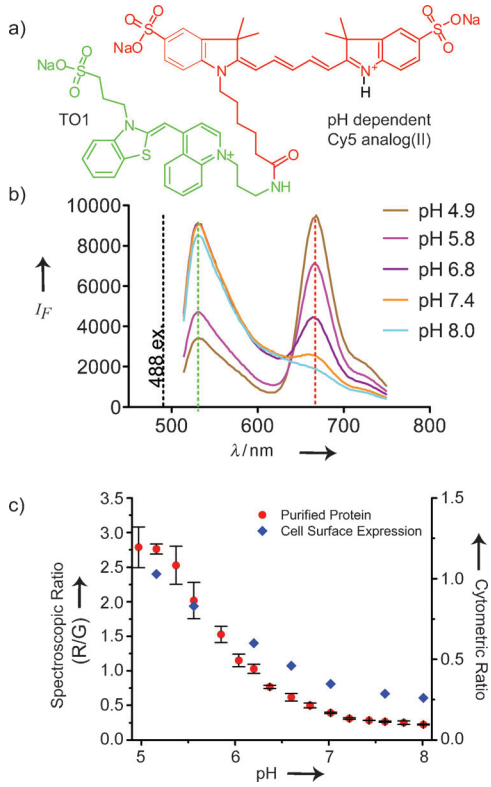
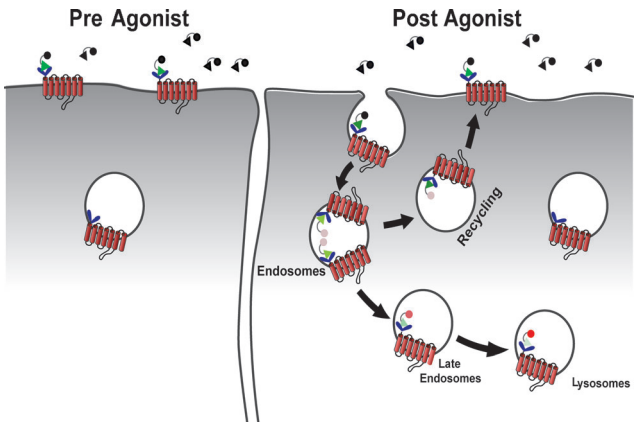


Figure 1. Structure and spectroscopic properties of the pH biosensor. a) The chemical structure of the FRET-based dye. A derivative of thiazole orange, TO1, is linked to the pH-dependent Cy5 analogue II. b) The fluorescence emission spectra of the FAP-bound dye in citrate/phosphate buffer at various pH values. Upon excitation at $\lambda = 488$ nm, energy transfer from the donor (TO1) to the acceptor (II) depends on the pH value. c) The pH-dependent ratio of red emission to green emission from the biosensor in solution and on cell surfaces.



Scheme 1. The operation of the biosensor. The FAP on the cell-surface protein activates the fluorogen in the media. Upon internalization, the FRET-based emission ratio of the biosensor can distinguish pH values in different compartments. Resident vesicles (e.g. golgi, and previously internalized receptors) are not labeled.

duction of 3T3 cells (NIH; see the Supporting Information) produced N-terminal-labeled β_2AR, with the FAP presented to the extracellular milieu.[19] Because of the low K_D value of the FAP–dye interaction, the concentration and activation of the dye by FAPs on the cell surface results in a substantially higher signal-to-background activation than the 10-fold seen in solution measurements [100-fold or higher as measured with line-scans (data not shown)]. The neutral pH of the media evokes minimal fluorescent response from II but bright fluorescence from the FAP-bound TO1 (Figure 2a). Upon agonist activation (isoproterenol, 10 μM) the adrenergic receptor internalizes and rapidly sequesters away from the plasma membrane into the endosomal pathway (see Figure S4 in the Supporting Information). Upon acidification of the vesicles, II is activated by protonation, thus enhancing the FRET-sensitized red emission (Figure 2b, and Movie S1).

Drug treatments demonstrated the biological relevance and specificity of this method. Acidification of vesicles was blocked with chloroquine[20] (a general cell permeant base) and bafilomycin A_1 (an ATPase inhibitor),[21,22] and trafficking was altered using Latrunculin A to block actin-dependent recycling of the β_2AR.[23,24] Alteration in the biosensor fluorescent ratio was recorded under the influence of these drugs (Figure 2 and see Figure S5 in the Supporting Information) and the mean vesicle pH value was estimated relative to the nigericin calibration data (Figure S6). Distinct pH phenotypes were seen after manipulation of endosomal acidity or receptor trafficking.

Dendritic cells (DCs) of the immune system are responsible for priming T-cell responses to protein antigens to generate adaptive immunity to pathogens.[25] Uptake of antigens is a critical first step in the process and DCs have multiple mechanisms for internalizing antigens, including phagocytosis, macropinocytosis, and receptor-mediated endocytosis.[26] In addition, DCs that display antigens internalized by neighboring cells, a phenomenon termed "cross-dressing", have been proposed as an amplification mechanism in generating effective immune responses.[27] The precise mechanism by which these cells achieve cross-dressing has not been determined. One proposed mechanism is transfer of cellular components between DCs, where a recipient cell engulfs considerable quantities of the plasma membrane of donor cells through intercellular nanotubules and other intercellular contacts, a process called trogocytosis.[28–30]

Critical questions in understanding the cross-dressing of DCs are whether whole protein antigens or peptide fragments are transferred between cells, and whether transfer occurs into acidified endocytic compartments within recipient cells, where further processing can occur, or through simple mixing of membrane proteins during cell–cell contact. To address these questions, we used the surface-displayed biosensor as a surrogate protein antigen that was transfected into human monocyte-derived DCs. The interaction between biosensor-expressing DCs and naïve DCs added to the culture was then visualized. As shown in Figure 3 and Movie S2 (see the Supporting Information), when pH-sensor-labeled dendritic cells (green signal) interact with a naïve cell, the biosensor transfer occurs almost exclusively into acidified endocytic compartments, thus resulting in an enhanced red signal from II. This enhancement demonstrates that an intact membrane protein (dye-bound biosensor) can be internalized directly into an endocytic compartment of adjacent cells, and that transfer does not result from the mixing of plasma membrane

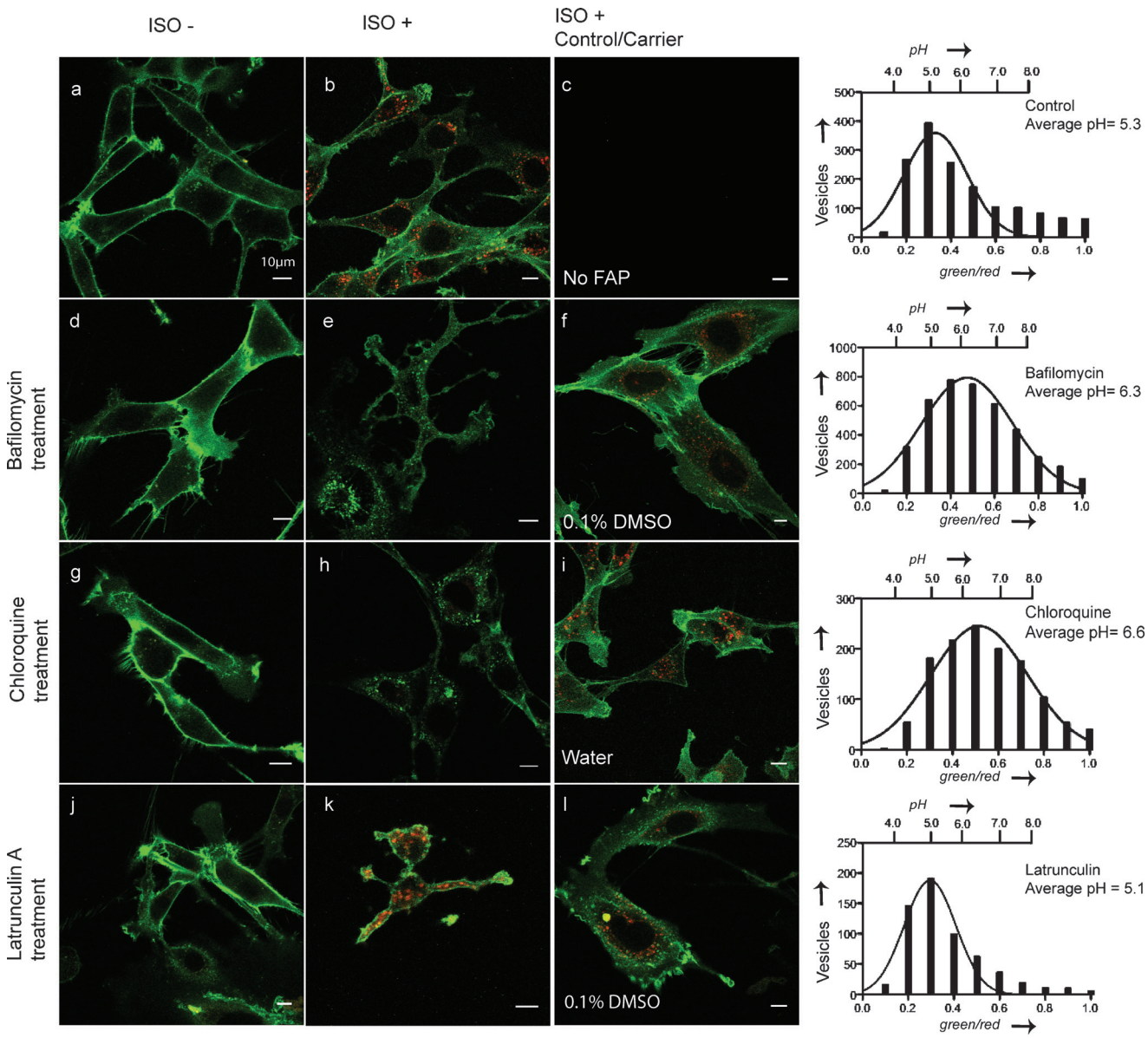


Figure 2. Tracking endocytosis using the targeted pH biosensor on β_2-adrenergic receptor. a) The surface labeling of the β_2AR with the biosensor dye. b) Upon adding the agonist (10 μM isoproterenol) to live cells, the receptors internalize in a matter of seconds and acidic vesicles are visible with enhanced red emission. c) Untransfected cells show no activation of the dye even in the presence of isoproterenol. d) Labeled cells pretreated with bafilomycin. e) Cells labeled in d) internalize neutral vesicles in response to the agonist. f) The carrier control. g) Cells treated with dye. h) Cells in g) treated with isoproterenol followed by chloroquine show neutralized vesicles. i) The carrier alone shows normal acidic vesicles. j) Labeled cells treated with Latrunculin A and dye. k) Cells in j) reveal a slight increase in acidity upon agonist treatment. Consistent with rerouting to the lower pH degradative pathway compared to the carrier control (l). The single vesicle ratio distribution from the cells exposed to these drugs is shown as a histogram.

during contact. In addition, this signal could not arise from direct transfer of endosomes or other vesicular structures containing the sensor, because these structures would be topologically incapable of binding to the dye in the media in transit to the recipient cell. Figure S7 and Movie S3 show transfer from FAP-transfected cells to naïve cells labeled with a cell-surface antibody, thus revealing transfer of vesicles to the recipient cell through nanotubules without extension of the donor cell membrane, and not a result of active endocytosis in the donor cell. These data support a model for antigen sharing between dendritic cells involving transfer of patches of intact plasma membrane from donor cells through an active endocytic process into recipient cells.

We have demonstrated a genetically targeted surface-selective pH biosensor that is useful for dynamic analysis of endosomal trafficking and antigen processing. This sensor is based on a genetically targeted fluorogen linked to a pH-sensitive dye that undergoes a significant change in FRET efficiency in response to environmental pH changes. Use of this biosensor to study receptor-mediated endocytosis in fibroblast cells revealed typical behavior, and indicated drug-induced alterations in the pH of the vesicles in living cells.

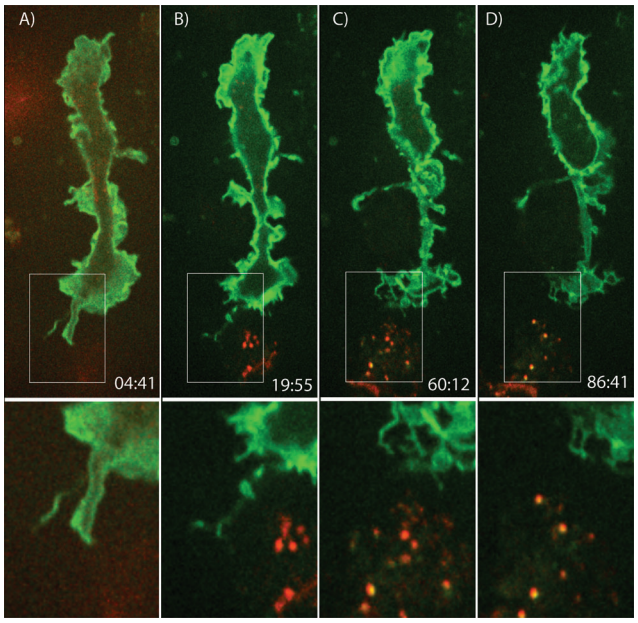


Figure 3. Antigen cross-talk and presentation in dendritic cells. Transfected DCs were cultured and labeled with the pH-sensitive fluorogen, and then contacted with untransfected DCs to track the fate of the pH sensor complex. a) The green color of the TO1 signal on the surface of the dendritic cells immediately after addition of untransfected DCs (top panel). The lower panel is the zoomed-in inset. b–d) Over time transfer of surface protein from the green cells to the unlabeled adjacent DC is seen, and accompanied by a shift of the reporter signal into the red, thus indicating transferred contents are in acidic compartments of the recipient cell.

Because the surface selection is achieved chemically, these measurements potentially extend to 3-dimensional tissue culture systems and live animals. The ability to target cellular pathways enables a number of studies with direct disease relevance.

The pH biosensor concept demonstrated here can be extended to a range of different indicators. Dyes that display a change in quantum yield, spectral properties, or extinction coefficient can be converted into targeted sensors that modulate the energy-transfer efficiency in response to analytes. Dyes can be made cell permeable, and cells can be treated to allow dye incorporation. Unlike direct fluorescent labeling, the fluorogen biosensor dye does not have to be washed away to reduce the background signal. This represents a significant new class of targeted biosensors for measuring local changes in the cellular environment.

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