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Plasticity of the asialoglycoprotein receptor deciphered by ensemble FRET imaging and single-molecule counting PALM

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Summary:
The plasma membrane contains numerous membrane protein receptors that assemble into homo- and hetero-oligomeric complexes. The precise stoichiometry and composition of these complexes is likely to substantially influence a receptor's activity, underscoring the need for methods to decipher receptor assembly state in the native environment of the cell.
Here, we present a visualization approach to clarify the structural / functional plasticity of membrane receptors, using as model system the prototypic two-subunit plasma membrane receptor, the asialoglycoprotein receptor. This receptor is known to bind and internalize diverse classes of desialyated glycoproteins, and is crucial for the clearance of clotting factors and platelets under specific pathological conditions. With advanced spectroscopic tools, including ensemble fluorescence resonance energy transfer (FRET) imaging and single-molecule counting photoactivated localization microscopy (PALM), we test whether cells display different oligomeric forms of the asialoglycoprotein receptor, what the subunit stoichiometries of these forms are, and whether the different oligomeric forms have distinct ligand specificities. Our findings reveal that plasma membrane receptors like the asialoglycoprotein receptor may be far more plastic and modular systems than previously thought.

Using fluorescence microscopy, we first demonstrated that expression levels of the two subunits of the asialoglycoprotein receptor (i.e., RHL1 and RHL2) modulate a cell's ability to bind different desialyated ligands (including asialofetuin [ASF] and lactoferrin [LTF]). While ASF-binding required both receptor subunits to be present on the plasma membrane, LTF-binding occurred

either when RHL1 was expressed alone or when RHL1 was expressed in higher abundance than RHL2. To examine, whether this differential ligand binding was due to receptor assembly into different homo- and hetero-oligomeric states (having different ligand specificities), we employed fluorescence resonance energy transfer (FRET), a radiation-less transfer of energy from an excited dye, the donor, to another nearby chromophore, the acceptor, used to determine inter- or intramolecular distances or molecular vicinities of 1 - 10 nm [1]. Our FRET approach permitted the quantitative analysis of receptor subunit assembly. Analyzing homo-oligomerization, we found that RHL1 self-associates extensively, while RHL2 does not, and instead distributes mainly as homo-dimers in the plasma membrane. This differential tendency to homo-associate was mediated by specific protein domains. Furthermore, the novel use of a photoactivatable fluorescent protein for FRET experiments suggested that the presence of RHL2 limits the extent of RHL1 homo-oligomerization, while the reverse is not true and RHL2 remained homo-dimeric. We then used FRET to examine RHL1 and RHL2 hetero-oligomerization. The FRET data in conjunction with an analytical model we generated suggested a receptor subunit stoichiometry of 2:1 (RHL1: RHL2) with a basic building block of 2x RHL1 dimers and 1x RHL2 dimer.

To obtain direct visualization of receptor composition and stoichiometry *in situ*, we used photoactivated localization microscopy (PALM) for single-molecule counting. In this single-molecule based imaging technique, repeated activation and sampling of individual molecules permits densely expressed fluorescent proteins to be resolved in time [2]. PALM is typically used to create superresolution images of cellular structures. Utilizing a rigorous new protocol for PALM that included spectrally distinct fluorophore calibrators, we showed this technique could also be used to reproducibly count single events corresponding to photoactivation of single fluorescent proteins. The counting calibrators, consisting of chimeras with distinct ratios of PAGFP and PAmCherry, ensured that activation and detection efficiency were constant during single molecule measurements. This made our counting of molecules reproducible, which is essential for obtaining quantitative information about the assembly and stoichiometry of membrane proteins in homo- and hetero-oligomeric states *in situ*, even in membrane areas of high receptor density. Using this approach, we visualized different co-existing receptor subunit assemblies, higher-order RHL1 homo-association, and RHL1 / RHL2 hetero-association into distinct clusters that preserve a 2:1 stoichiometry in the presence of free RHL2 subunits. Moreover, we showed that addition of exogenous ligands shifts co-existing receptor states at the plasma membrane of a cell, leading to the preferential internalization of one receptor complex over another.

In conclusion, we demonstrate that the two subunits of asialoglycoprotein receptor can assemble into both homo- and hetero-oligomeric complexes, displaying different forms with distinct ligand specificities that co-exist on the plasma membrane and can be modulated by exogenous ligands. The co-existence of distinct oligomerization states of the asialoglycoprotein receptor may

provide a versatile means to efficiently bind different glycoproteins as they suddenly arise, for instance, in septic conditions. The plastic modularity of the receptor system, on the other hand, may allow rapid shifts in specificity by changing the relative abundance of its different oligomeric states and account for the rapid depletion of thrombogenic material. In turn, the variety of potential ligands for asialoglycoprotein receptor and the propensity of its subunits to form distinct oligomers may explain previous inconsistent biochemical results regarding receptor stoichiometry, underscoring the importance of deciphering oligomerization in the single cell and on the single-molecule level.

Figure Caption:

(A) Expression and relative abundances of the two receptor subunits RHL1 and RHL2 modulate a cell's ability to bind different ligands, such as Asialofetuin (ASF) and Lactoferrin (LTF). For ASF-binding, both receptor subunits need to be expressed, for LTF-binding, however, the expression of RHL1 alone or in high abundance is sufficient.

(B) Ensemble FRET imaging combined with mathematical modeling helps dissect receptor subunit oligomerization and stoichiometry. Exemplarily shown are image acquisition of fluorescence in the donor-, acceptor- and FRET channel, data analysis of normalized FRET efficiency values in each image pixel, and mathematical modeling of receptor subunit hetero-oligomerization.

(C) Single-molecule counting PALM directly visualizes receptor subunit assembly and stoichiometry at the single molecule level *in situ.* Exemplarily shown are sequential single molecule image acquisition in a green and a red channel, standardization of single-molecule counting for assessing relative expression and stoichiometries by genetically encoded spectrally distinct calibrators, and a final quantitative single-molecule counting PALM image of receptor subunit hetero-oligomerization.

(D) The combined use of advanced spectroscopic methods results in a model of different co-existent receptor oligomers determined by receptor subunit oligomerization tendency, relative subunit abundances and presented ligands and defines plasticity of the two-subunit receptor system.

References:

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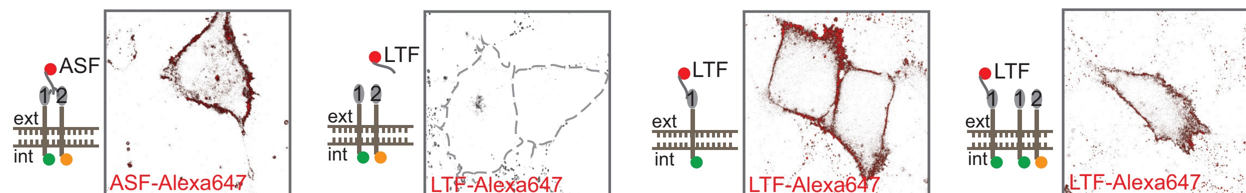
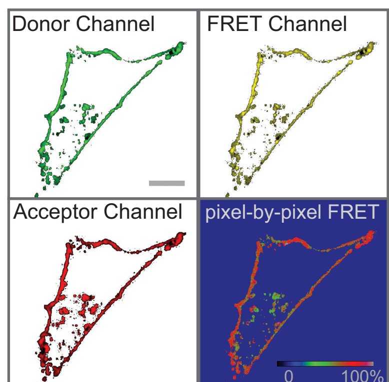
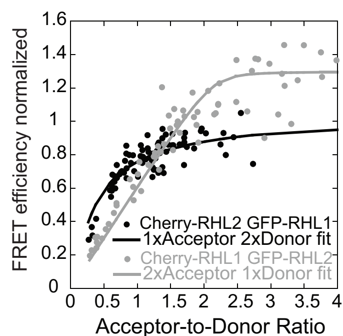
2. Betzig, E., et al., *Imaging intracellular fluorescent proteins at nanometer resolution.* Science, 2006. **313**(5793): p. 1642-5.

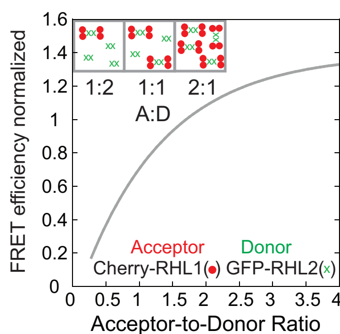
A Differential ligand binding



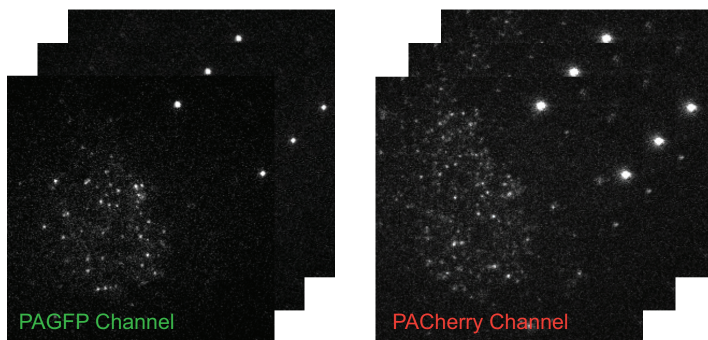
B ensemble FRET imaging

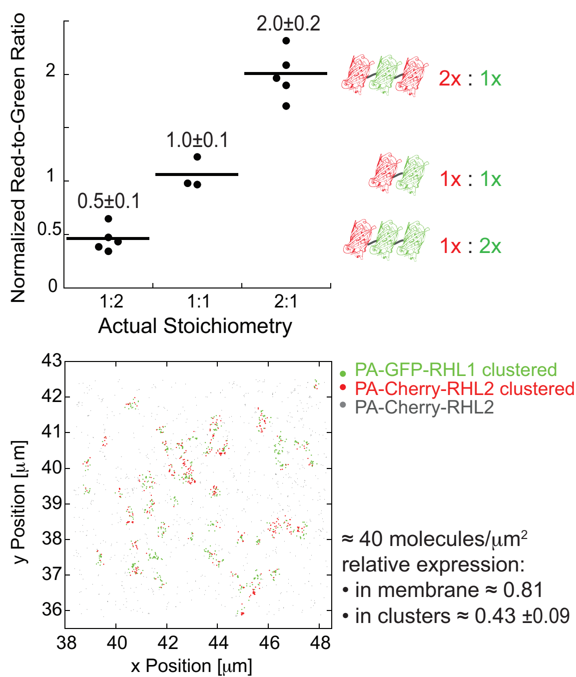






C single-molecule counting PALM





D Model of receptor plasticity