iBio Seminars Part II:
Looking for functional rafts in cell membranes

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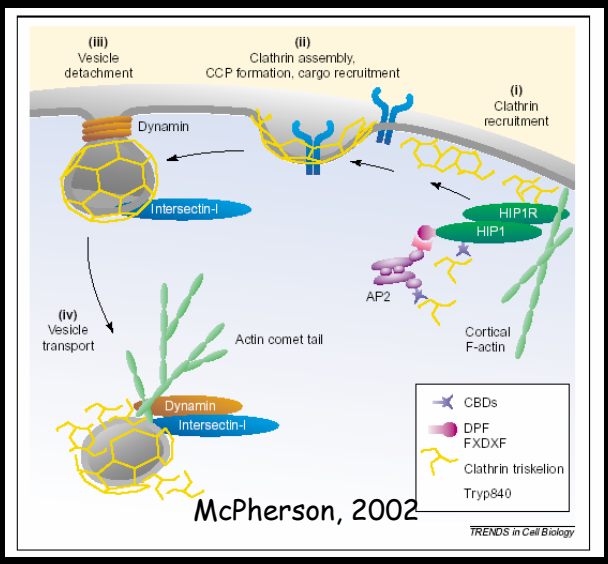
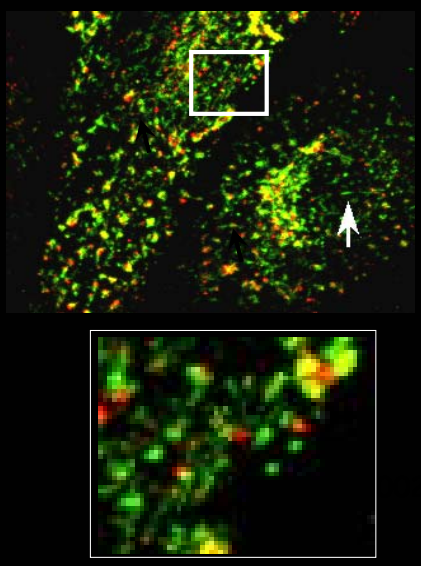
Clathrin/Dynamin-dependent endocytosis

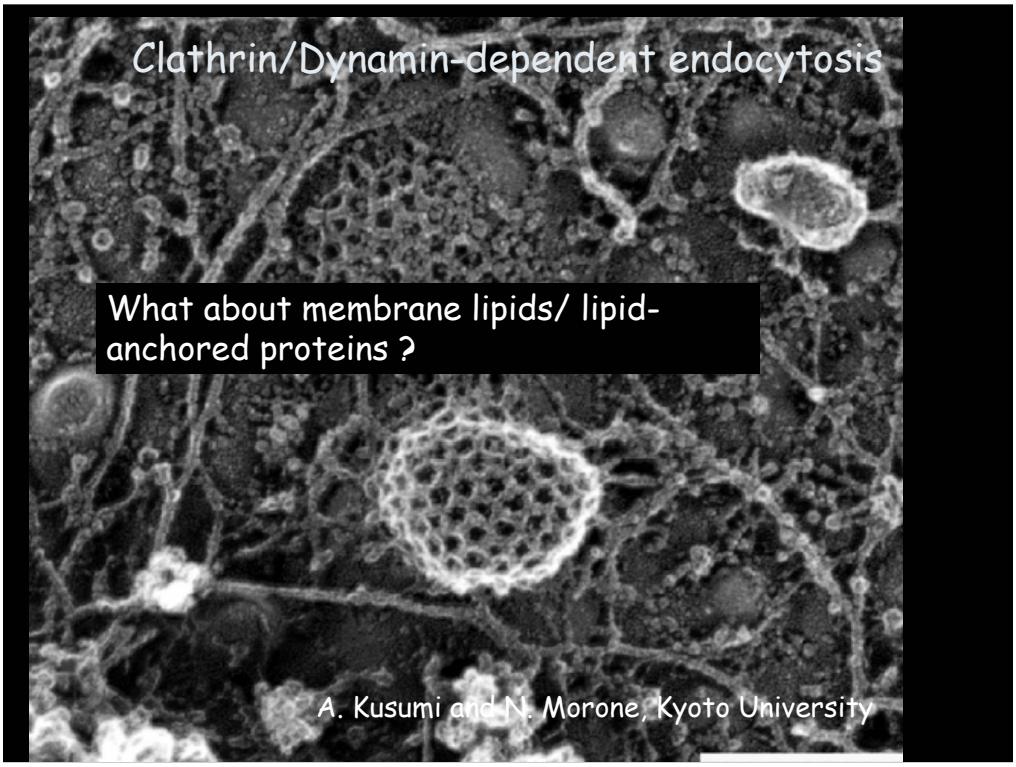


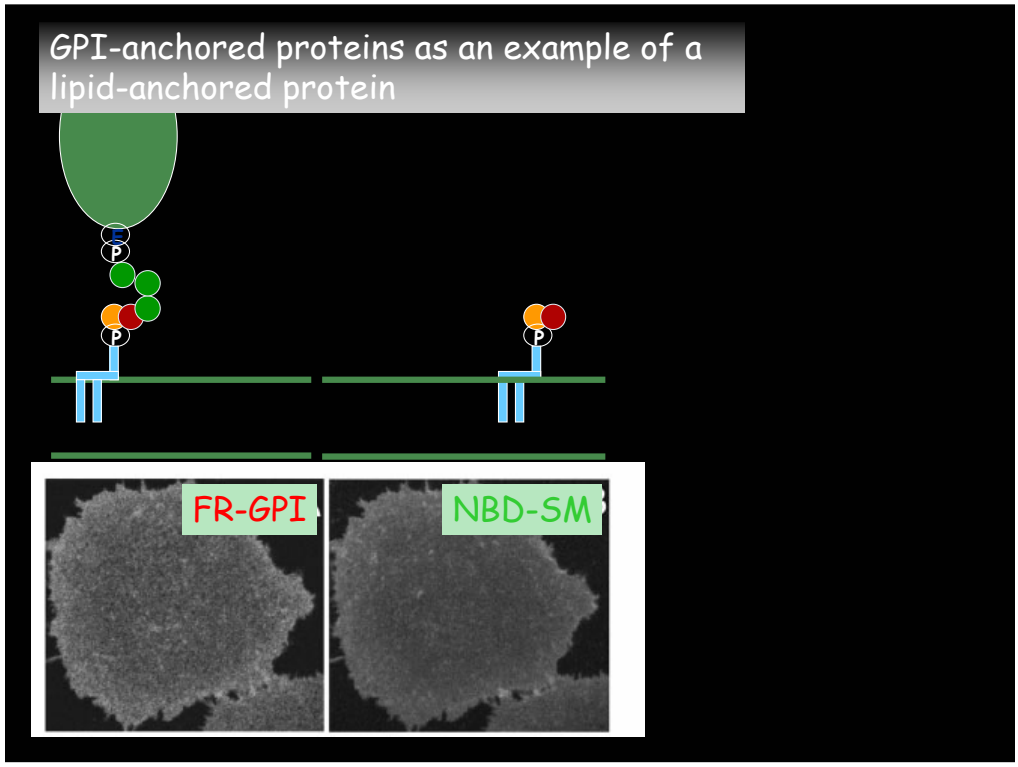
McPherson, 2002

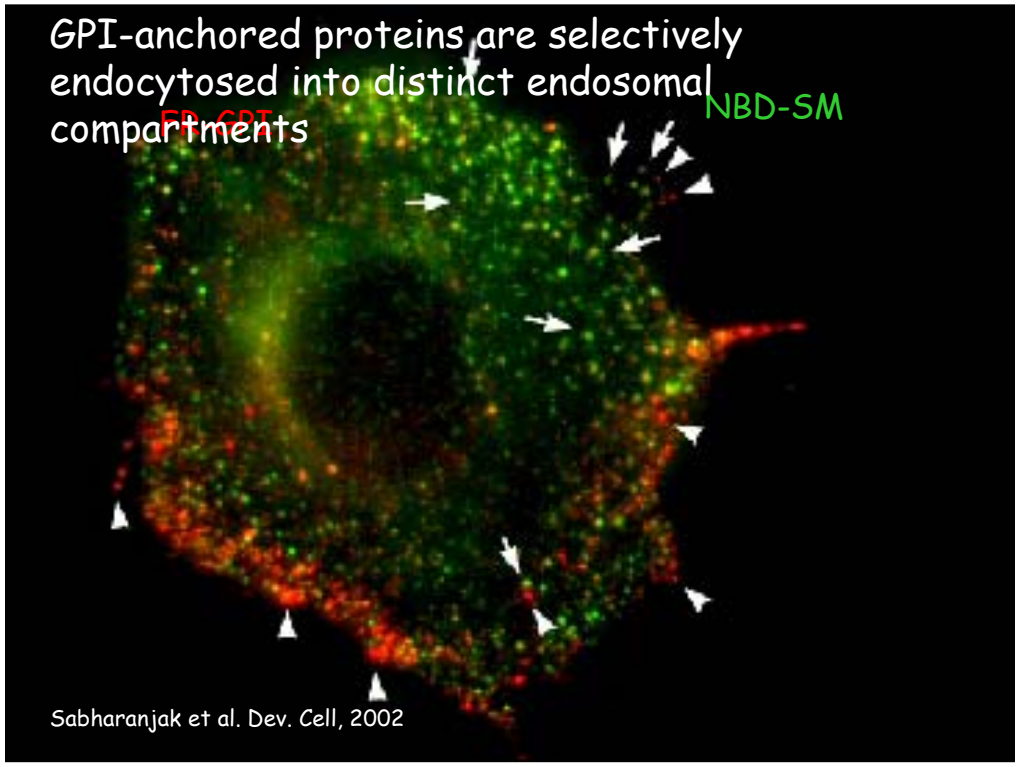
Clathrin/Dynamin-dependent endocytosis



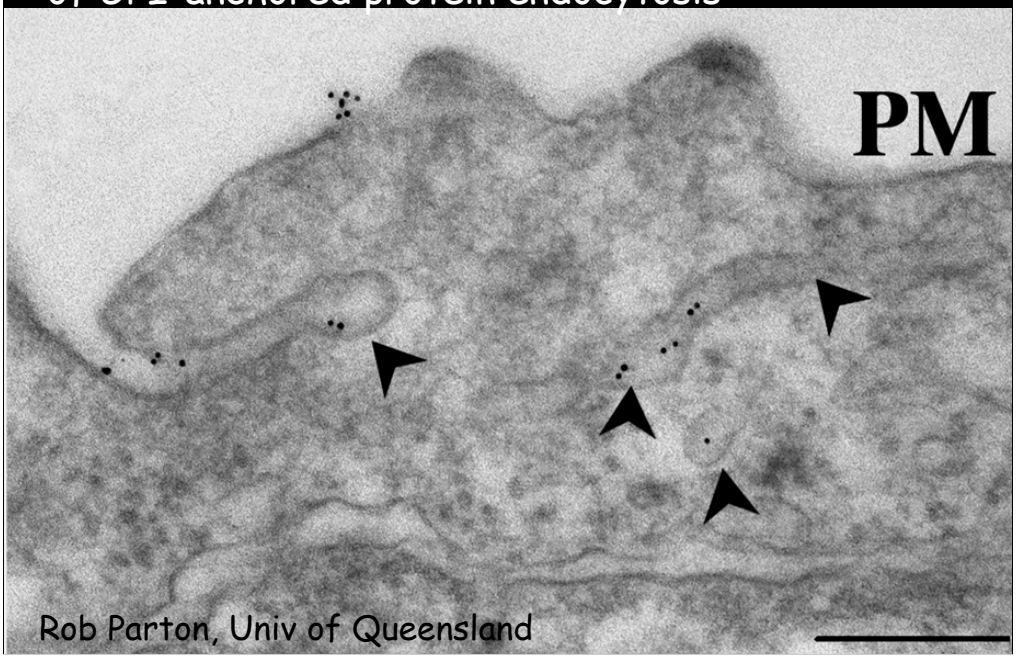
Clathrin/Dynamin-dependent endocytosis
A. Kusumi and N. Morone, Kyoto University


Clathrin/Dynamin-dependent endocytosis
What about membrane lipids/ lipid-anchored proteins ?
A. Kusumi and N. Morone, Kyoto University


GPI-anchored proteins as an example of a lipid-anchored protein
P
P
P
FR-GPI
NBD-SM

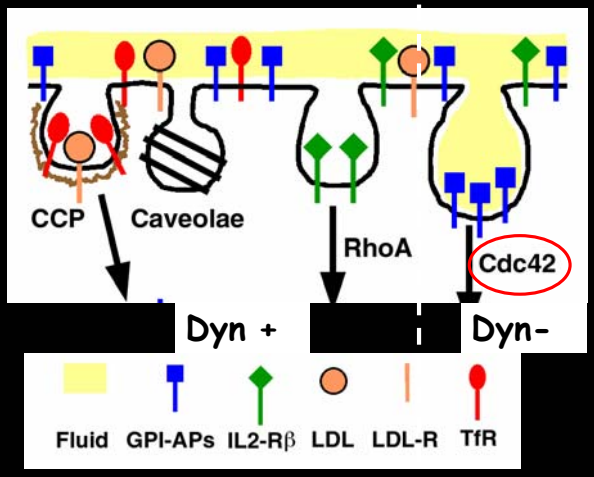

GPI-anchored proteins are selectively endocytosed into distinct endosomal compartments
FR-GPI
NBD-SM
Sabharanjak et al. Dev. Cell, 2002

High resolution EM- thick section images of GPI-anchored protein endocytosis



Rob Parton, Univ of Queensland

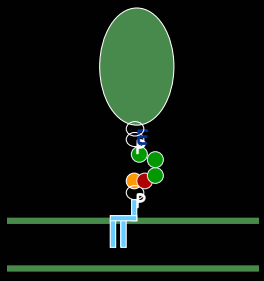
GPI-anchored proteins are endocytosed via a specialized pathway ' the GEEC pathway'



- Sabharanjak, Sharma et al. *Dev. Cell* (2002)
- Mayor and Riezman, *Nature Reviews* (2004)
- Kirkham et al. *J. Cell Biol.* (2005)
- Kalia et al. Mol. Biol. Cell (2006)
- Chadda et al. (2007)

GPI-anchored proteins are internalized via an pinocytic pathway that is

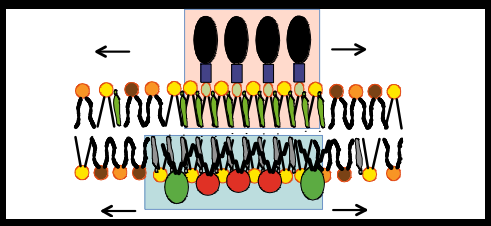
- Constitutive
- Lipid-selective
- Lipid-dependent
- Cdc42-regulated
- Actin-dependent



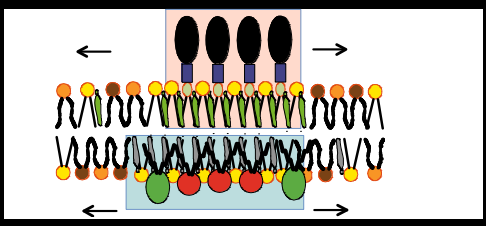
Does not utilize
- Dynamin
- Clathrin/ Caveolin
- Arf6
- RhoA
- Rac1

Membrane Rafts?

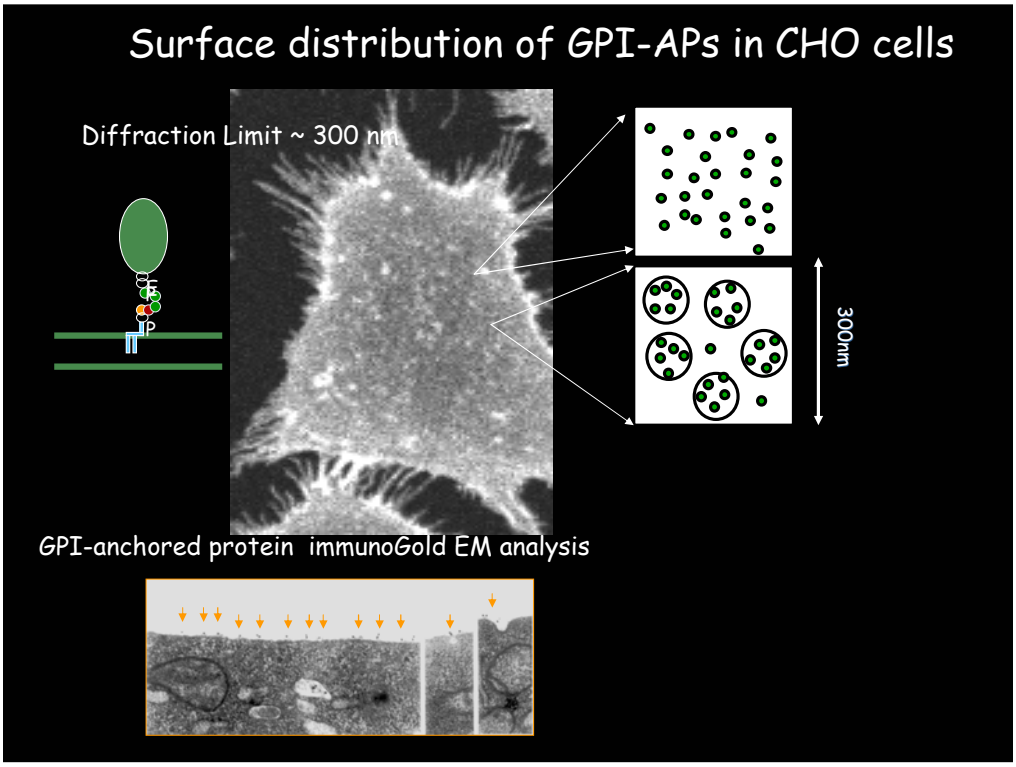
- dynamic clustering of cholesterol and sphingolipids to form domains in membranes
- platforms for the segregation of specific proteins responsible for sorting and signaling function



Looking for rafts



- detergent insolubility
- diffusion measurements
- proximity-based methods


Surface distribution of GPI-APs in CHO cells
Diffraction Limit ~ 300 nm
300nm
GPI-anchored protein immunoGold EM analysis

Proximity-based methods to examine segregation of GPI-anchored proteins

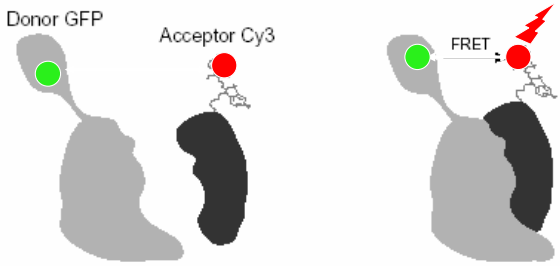
EM analysis (Mayor et al, *Science*, 1994; Prior et al, *JCB* 2003; Madore et al , *EMBO J* 2002)

Chemical crosslinking *in situ* ~ microdomains:
(Friedrichson and Kurzchalia, *Nature* 1998; Paladino et al, *JCB*, 2004)

Forster's Resonance Energy Transfer (FRET)
(Varma and Mayor, *Nature* 1998; Kenworthy et al., *JCB*, 1998; Sharma et al, *Cell*, 2004)

The FRET scale- A spectroscopic ruler

Ro ~ 1 – 10 nm



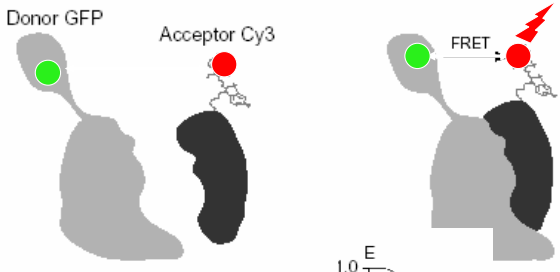
$$E = [1/\{1 + (r/R_o)^6\}]$$

$$R_o = 8.79 \cdot 10^{-5} [n^{-4} \cdot Q \cdot \kappa^2 \cdot J(\lambda)]^{1/6} \text{ Å}$$

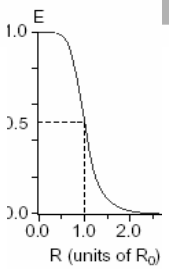
$$\kappa^2 = [\cos\theta_T - 3\cos\theta_A \cdot \cos\theta_D]$$

The FRET scale- A spectroscopic ruler

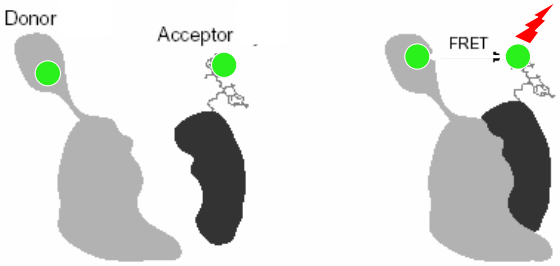
Ro ~ 1 – 10 nm



Forster's radius
~ 1- 8 nm for organic fluorophores

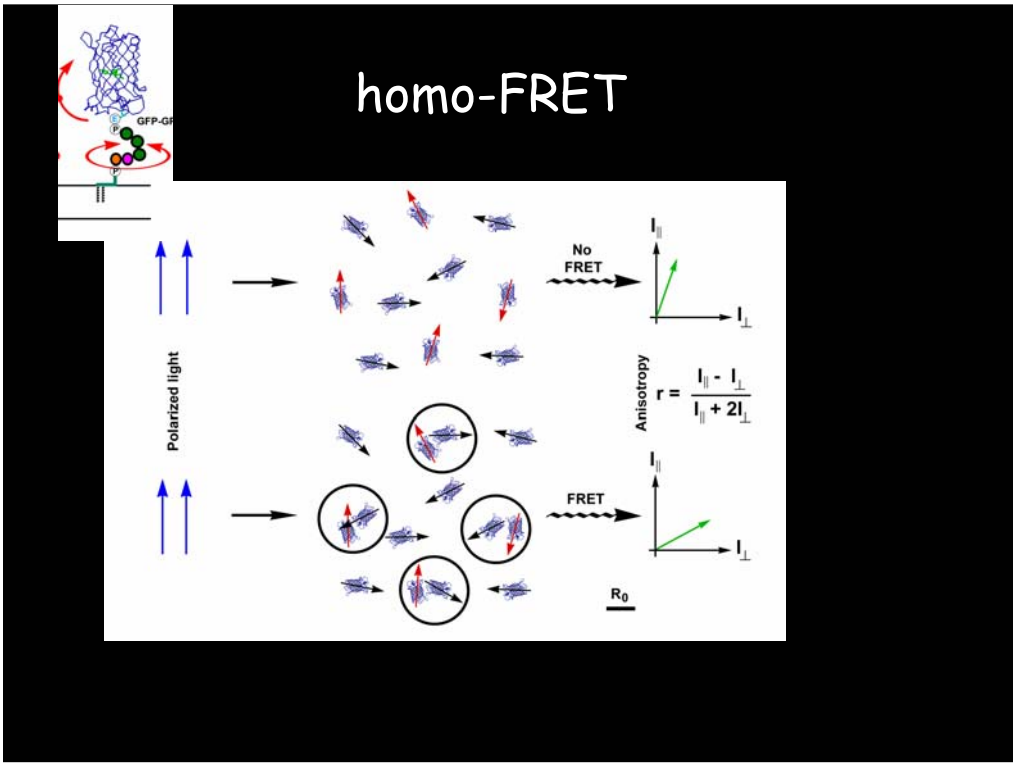


homo-FRET Depolarization FRET

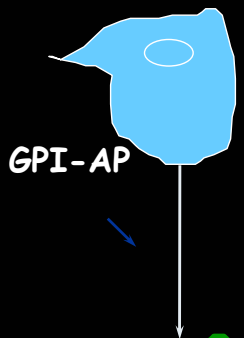


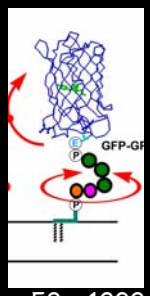
measure fluorescence anisotropy

Varma and Mayor, *Nature* (1998)


homo-FRET
GFP-GP
Polarized light
No FRET
FRET
Anisotropy
$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$
$I_{\parallel}$
$I_{\perp}$
R_0

Transfect CHO cells



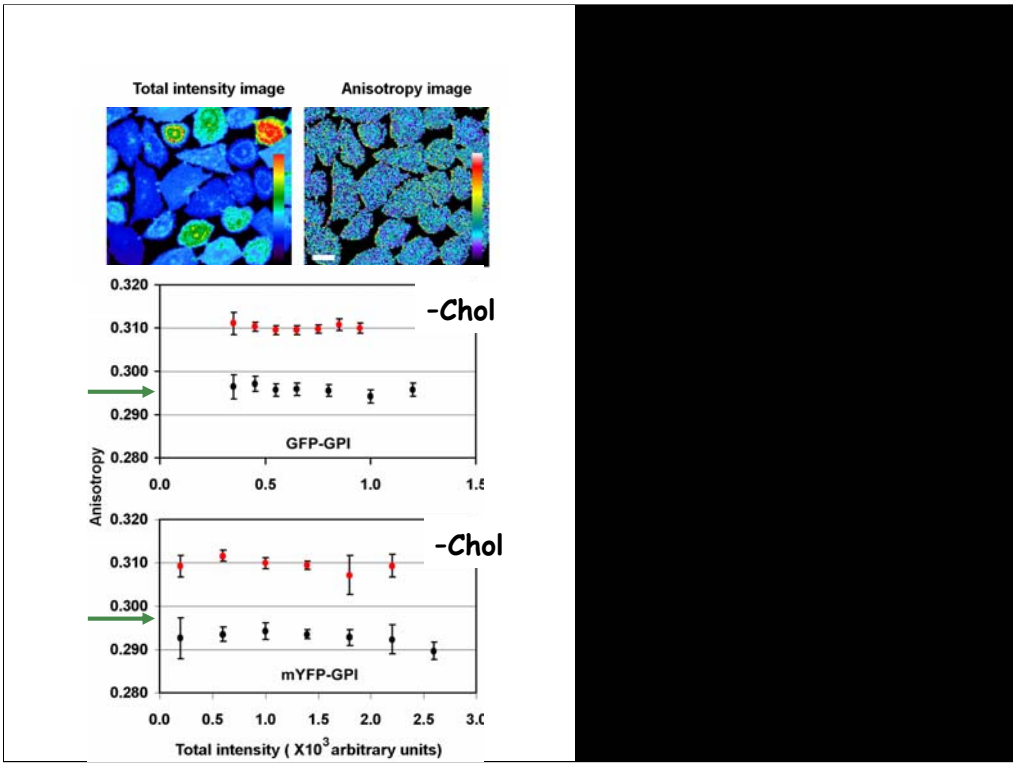


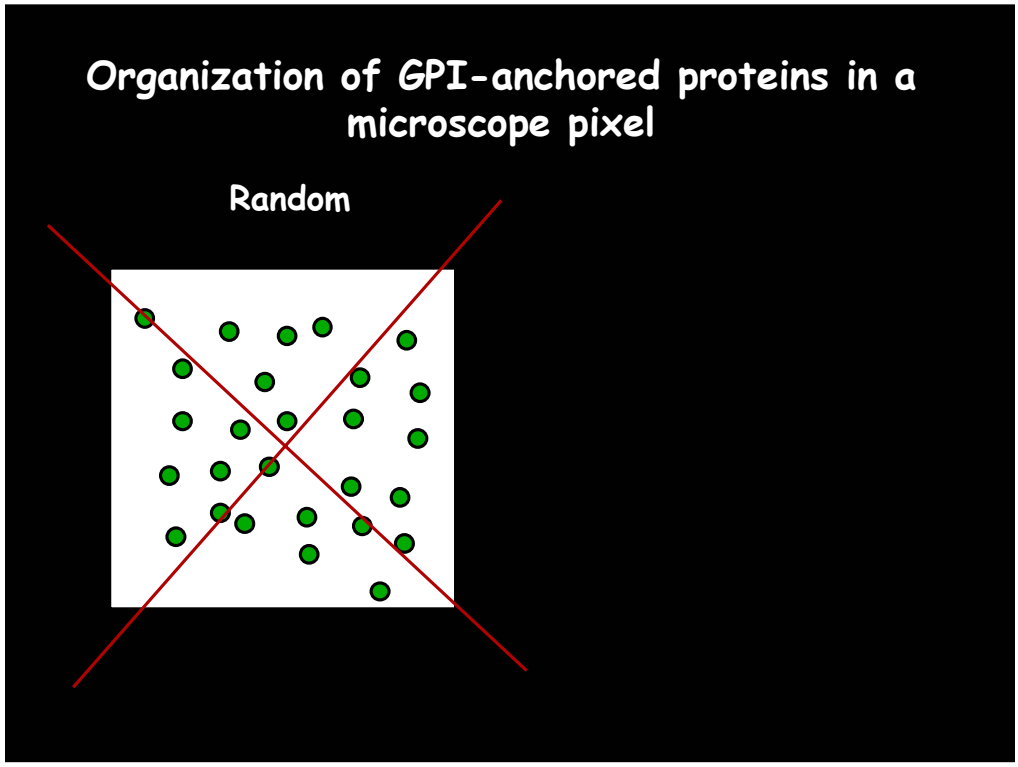
~ 50 - 1000 /um^2

GFP-GPI
mYFP-GPI
FR-GPI

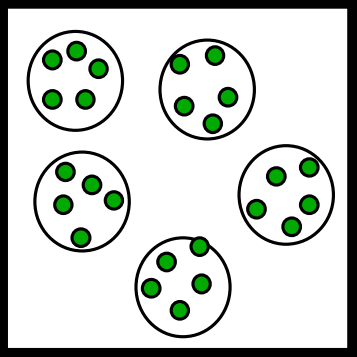
Measure anisotropy

(Varma and Mayor, 1998; FR-GPI)


Total intensity image
Anisotropy image
-Chol
GFP-GPI
-Chol
mYFP-GPI
Anisotropy
0.320
0.310
0.300
0.290
0.280
0.0
0.5
1.0
1.5
2.0
2.5
Total intensity (X10^3 arbitrary units)


Organization of GPI-anchored proteins in a microscope pixel
Random

Organization of GPI-anchored proteins in a microscope pixel

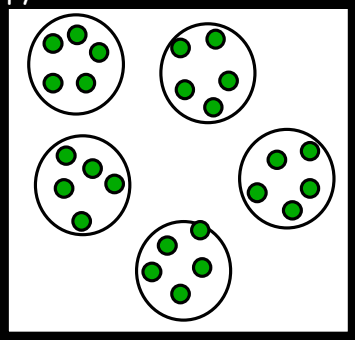


300 nm

(small clusters)

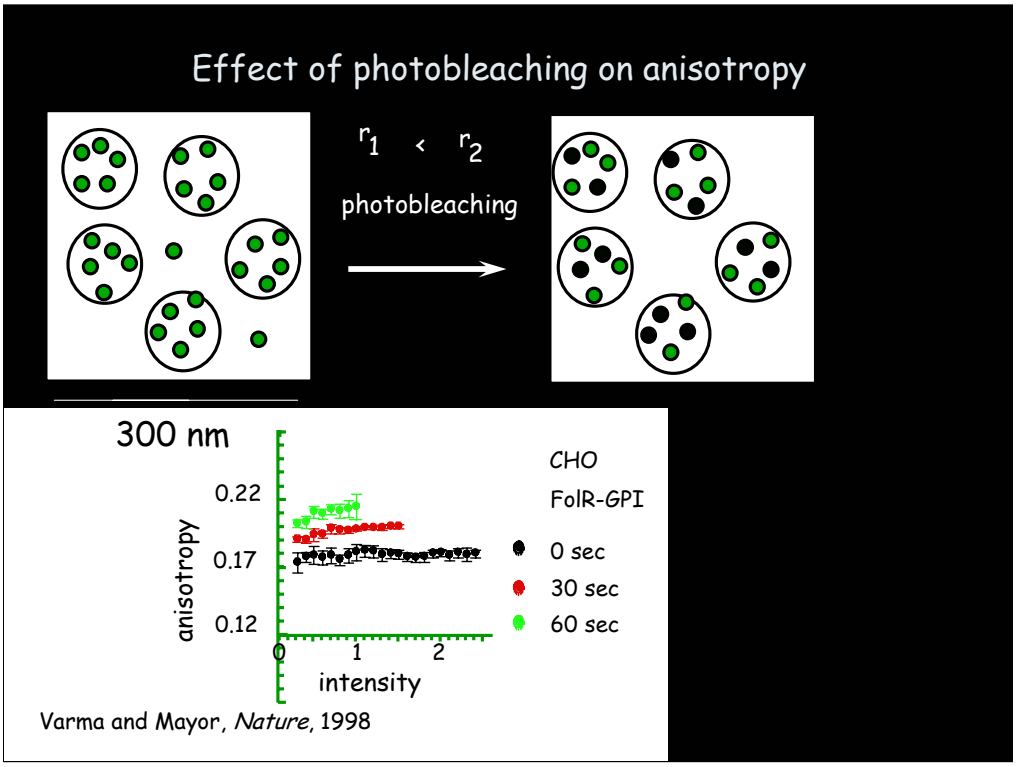
Organization of GPI-anchored proteins in a microscope pixel

high density $\leq$ 4 nm separation
From Time Resolved Fluorescence anisotropy measurements

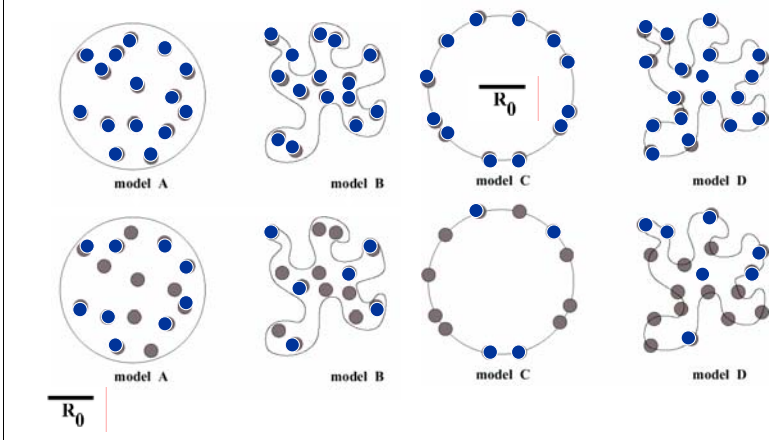


300 nm

(small clusters)

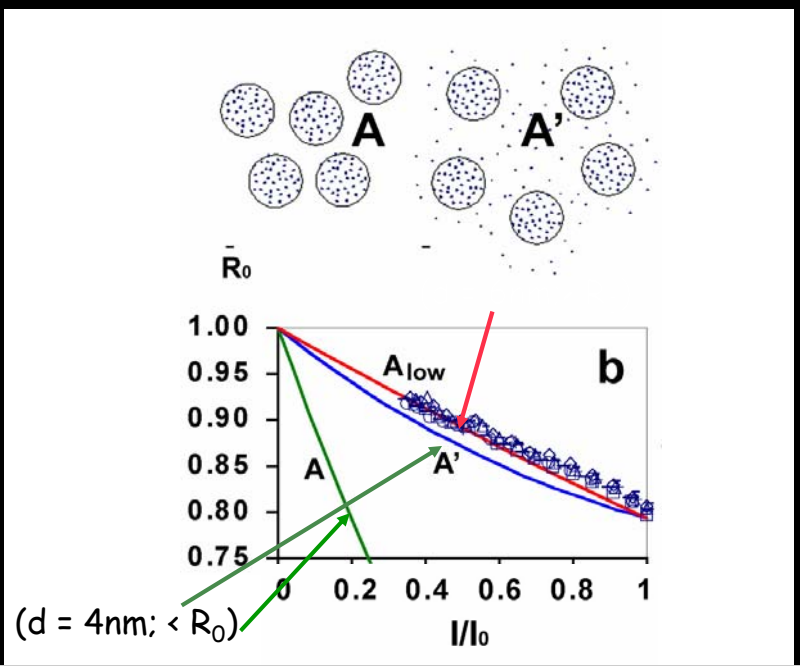

Effect of photobleaching on anisotropy
$r_1 < r_2$
photobleaching
300 nm
anisotropy
0.22
0.17
0.12
0
1
2
intensity
CHO
FolR-GPI
0 sec
30 sec
60 sec
Varma and Mayor, *Nature*, 1998

Possible architecture of domains

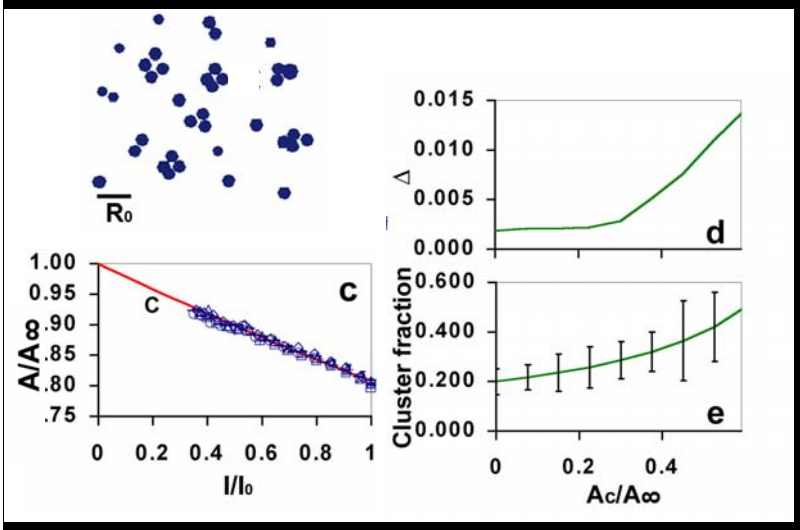


- change of anisotropy after photobleaching
- theoretical models

Possible architecture of domains in living cells?



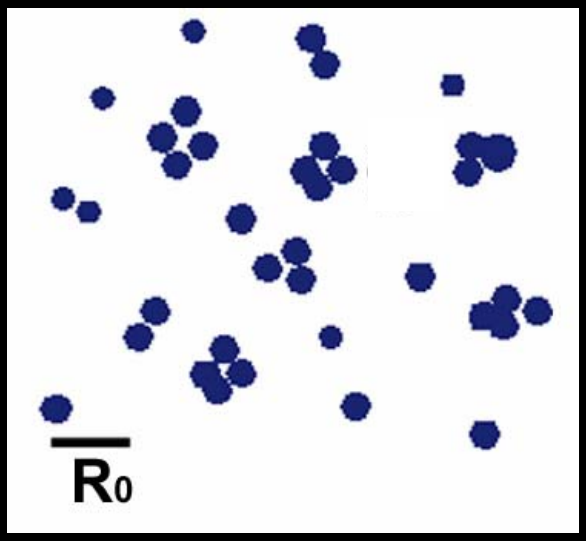
Structure of the FRET-species



(Data taken from cells with 20 fold different protein density)

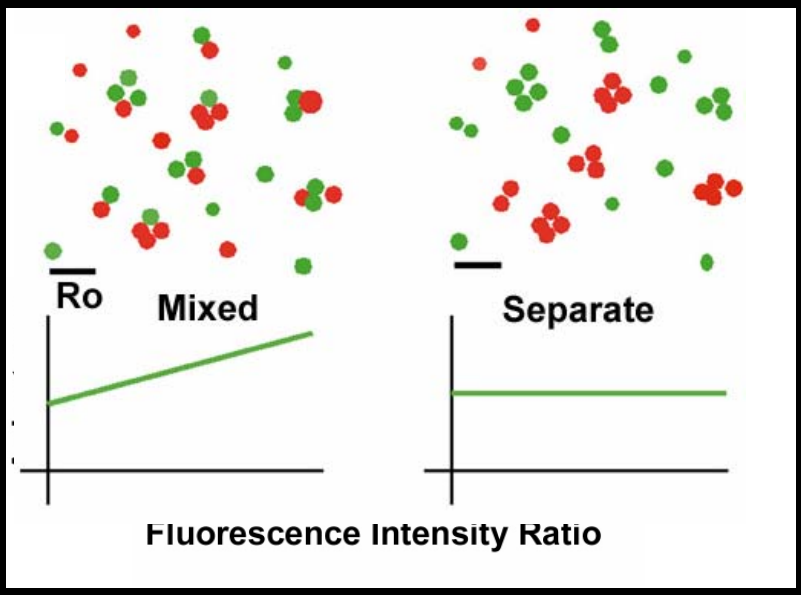
Sharma, Varma, Sarasij et al., *Cell* 2004

Mainly monomers and 20-40 % as nano-clusters –

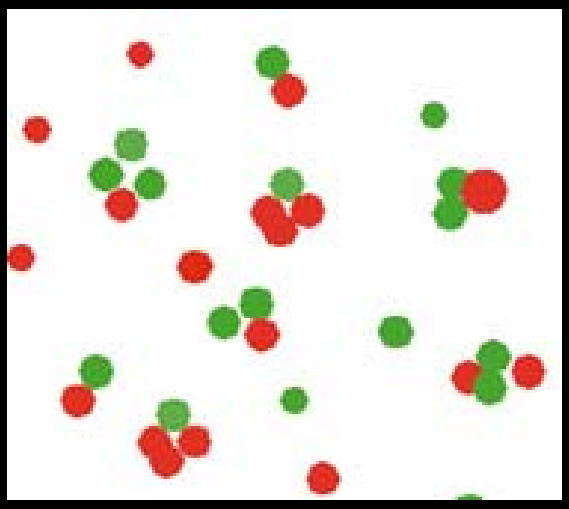


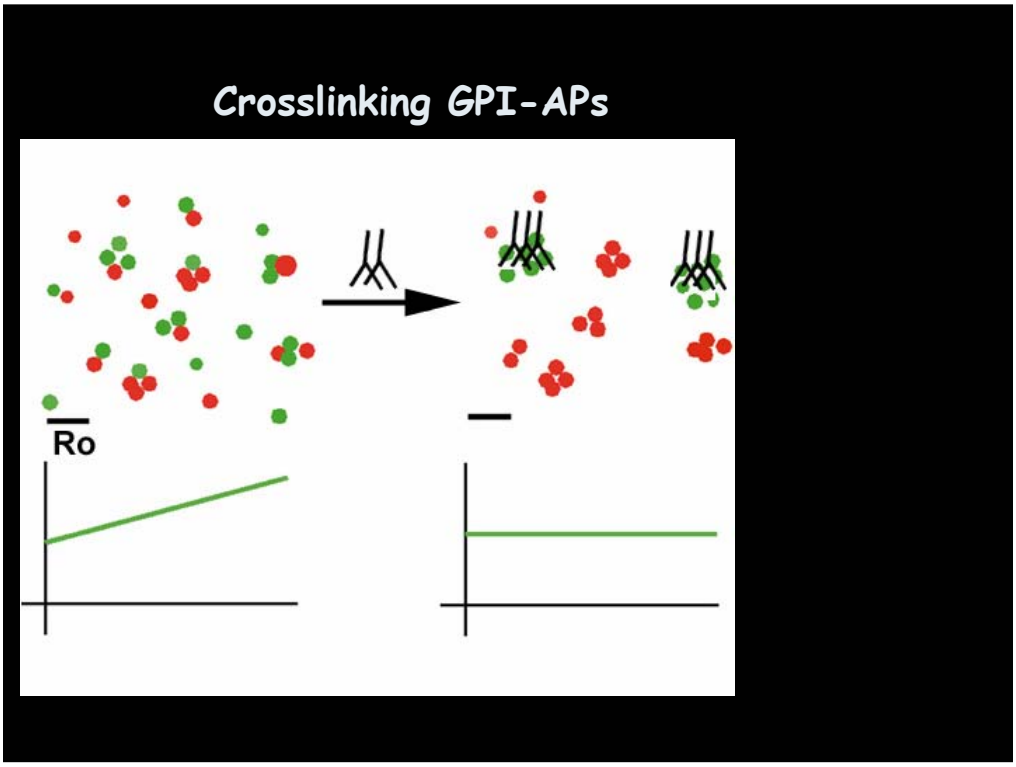
Sharma, Varma, Sarasij et al., *Cell* 2004

Do multiple GPI-APs *cohabit?*

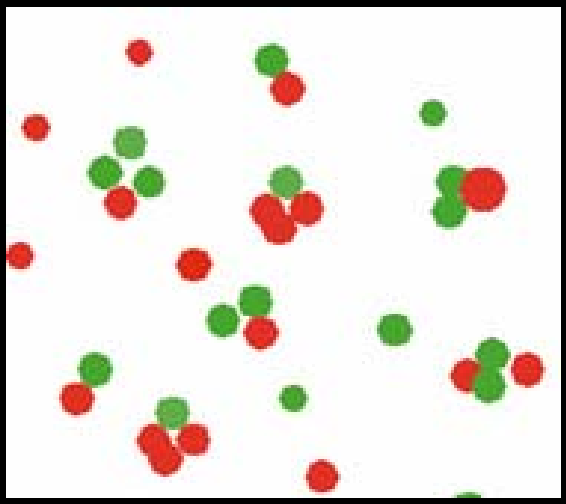


GPI-APs are organized as monomers and mixed nano-clusters

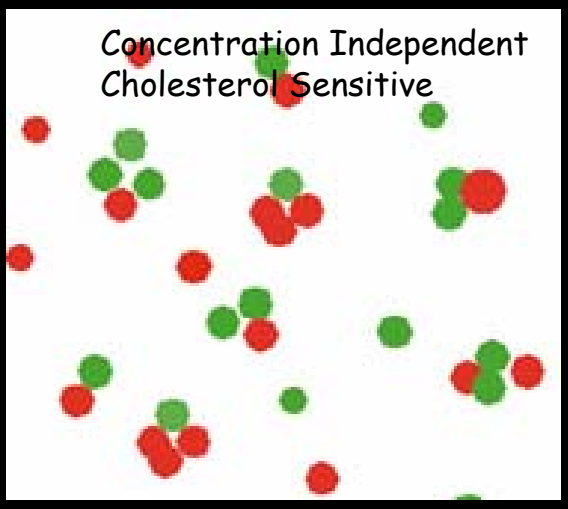



Crosslinking GPI-APs
Ro

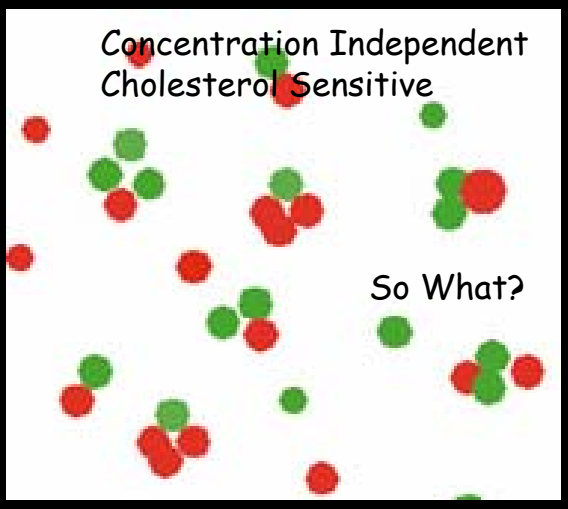
GPI-APs are flexibly organized as monomers and mixed nano-clusters



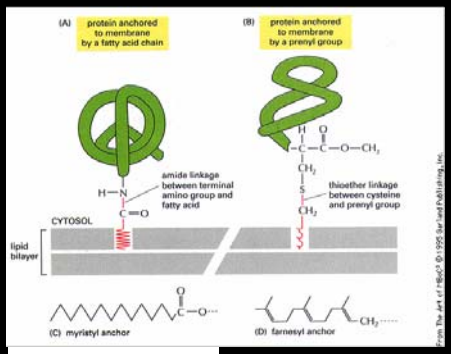
GPI-APs are flexibly organized as monomers and mixed nano-clusters

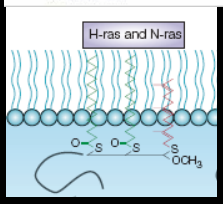


GPI-APs are flexibly organized as monomers and mixed nano-clusters

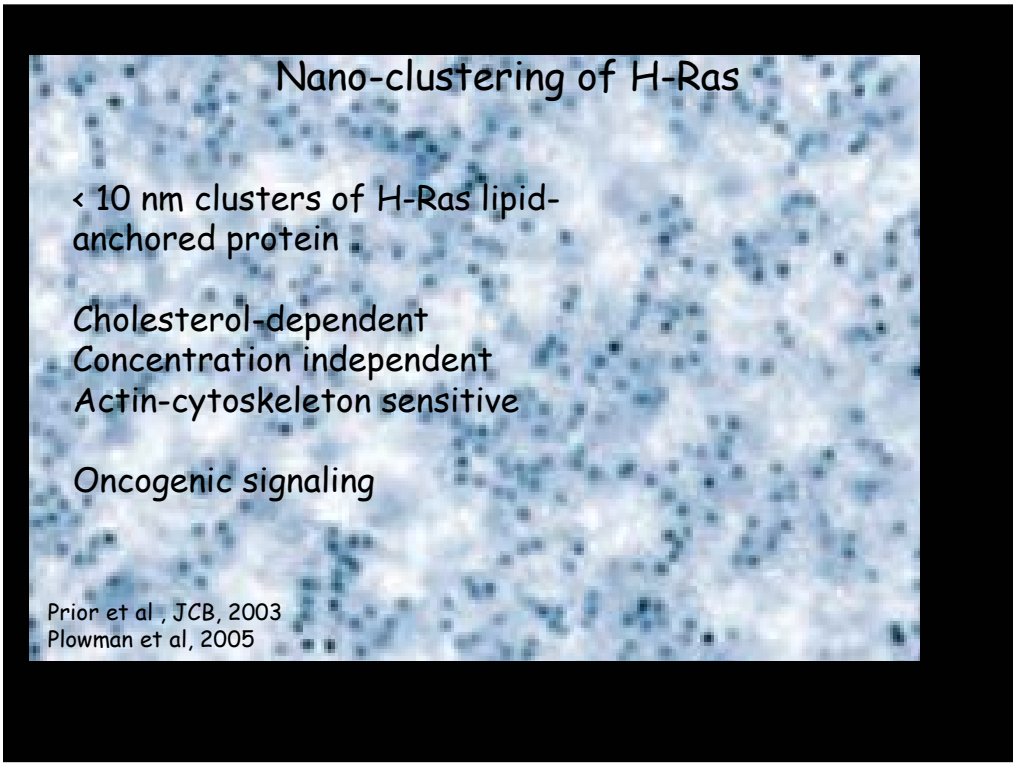


Organization of lipid-anchored proteins in plasma membranes

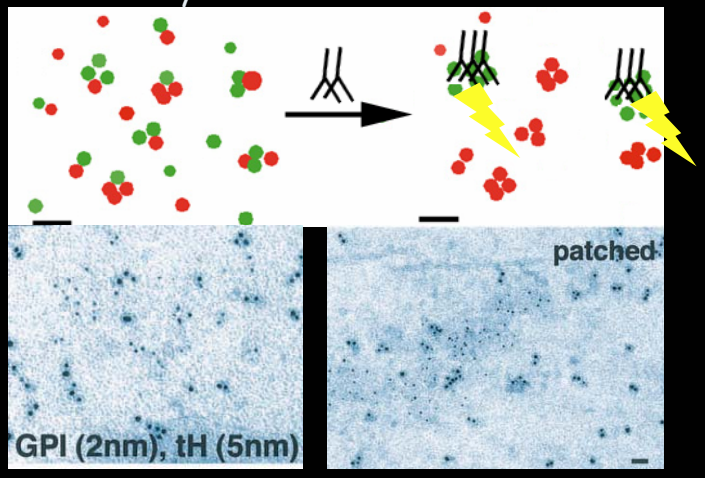




(Hancock, *Nature Reviews (MCB)*, 2003)


Nano-clustering of H-Ras
< 10 nm clusters of H-Ras lipid-anchored protein
Cholesterol-dependent
Concentration independent
Actin-cytoskeleton sensitive
Oncogenic signaling
Prior et al , JCB, 2003
Plowman et al, 2005

Crosslinking GPI-anchored proteins induces H-Ras-domains to coalesce suggesting a trans-bilayer connection



Prior et al , JCB, 2003

Other (glyco)lipids also show a clustered distribution

Ganglosides GM1 and GM3 exhibit a similar nano-clustered distribution

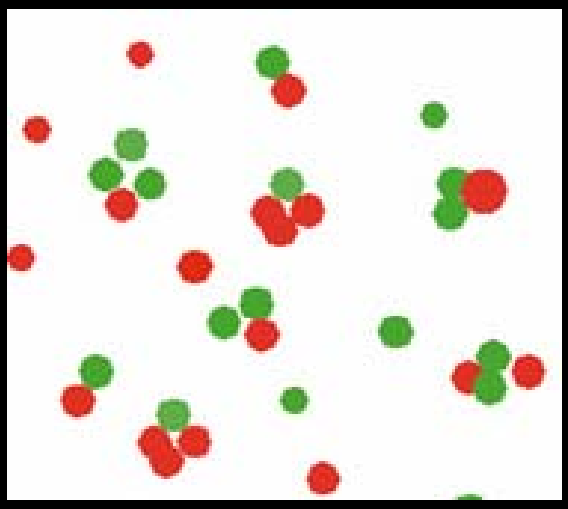
sensitive to cholesterol depletion

cold temperatures

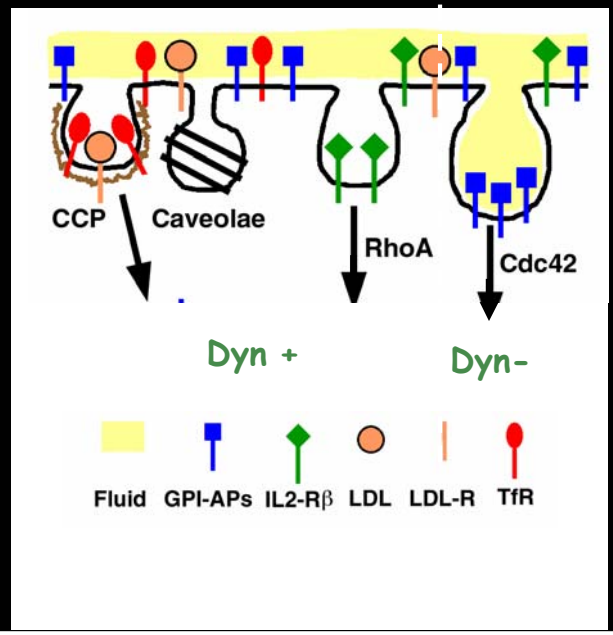
concentration independent

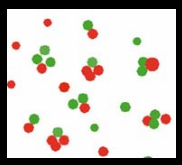
- Fujita, et al. MBC (2007)

Lipid-anchored proteins and lipids are flexibly organized as monomers and nano-clusters

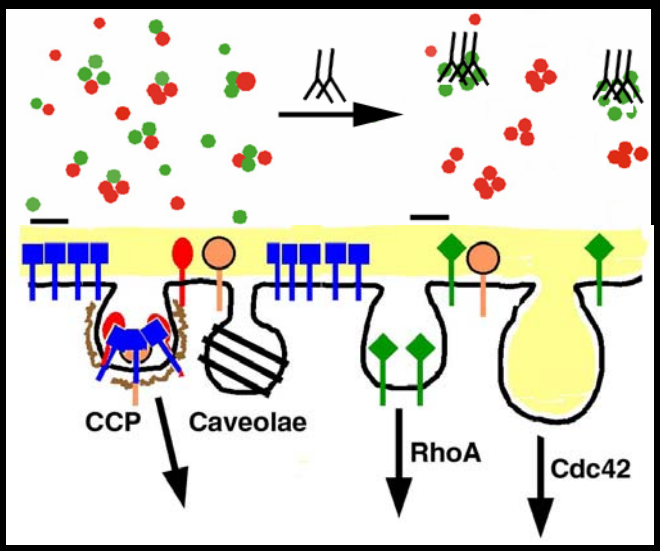


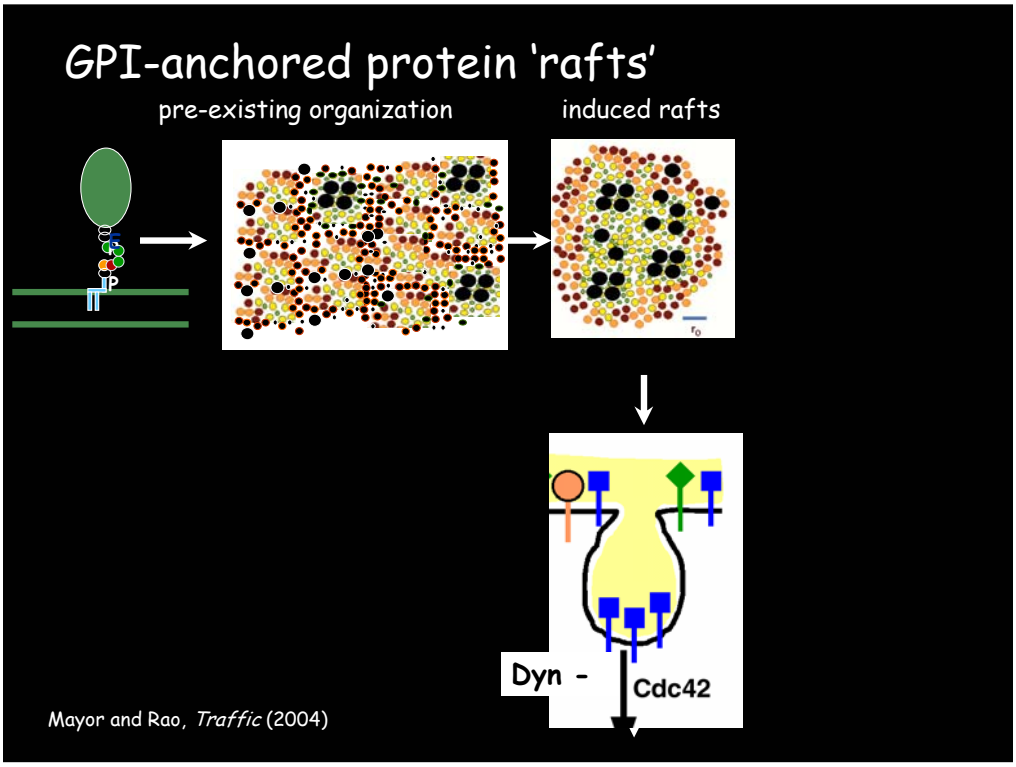
Endocytosis of GPI-anchored proteins and nanoclusters?

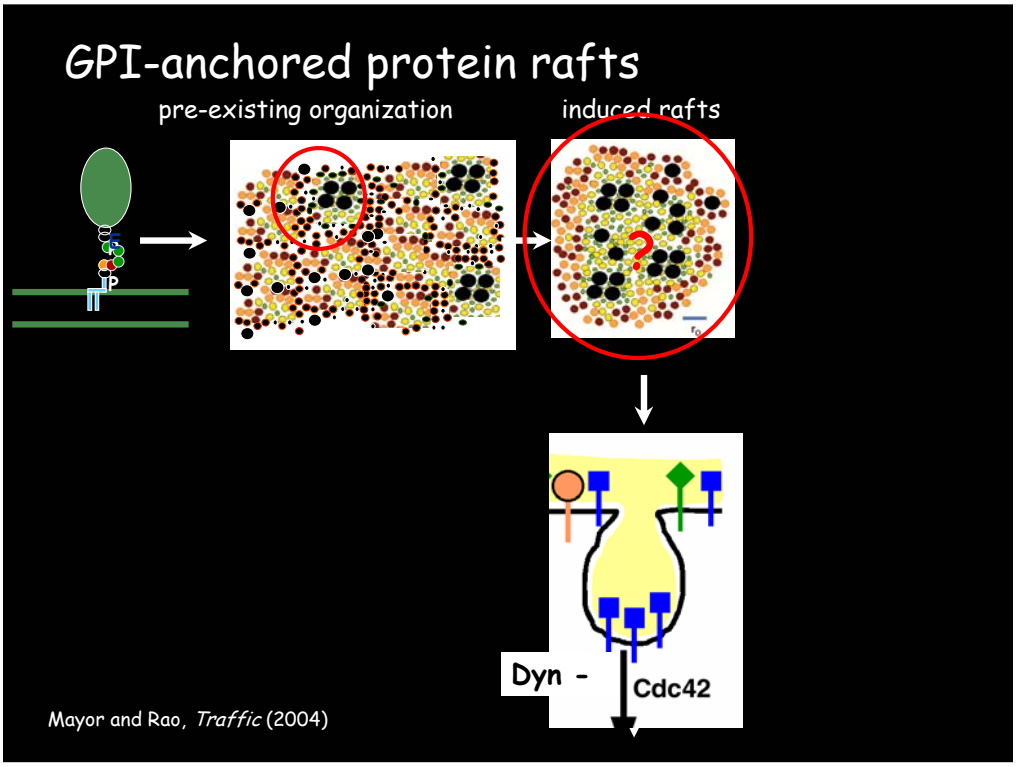




Crosslinked GPI-anchored proteins are not endocytosed via the GEEC-pathway



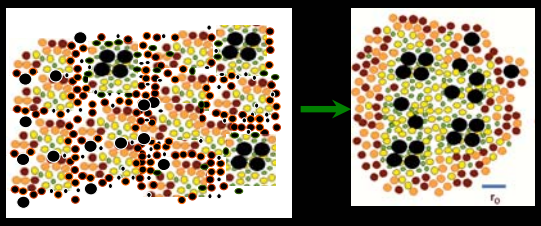

GPI-anchored protein 'rafts'
pre-existing organization
induced rafts
Dyn -
Cdc42
Mayor and Rao, *Traffic* (2004)


GPI-anchored protein rafts
pre-existing organization
induced rafts
?
Dyn -
Cdc42
Mayor and Rao, *Traffic* (2004)

Functional Rafts : A working hypothesis

pre-existing organization

induced rafts



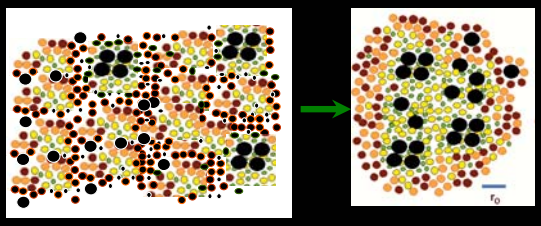
Sorting or Signaling Function

Mayor and Rao, *Traffic* (2004)

Functional Rafts : A working hypothesis

pre-existing organization

induced rafts



Sorting or Signaling Function

What makes nano-clusters?

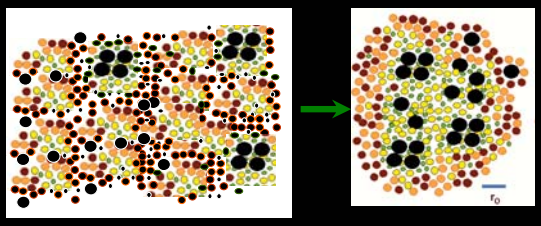
What induces functional domains?

Mayor and Rao, *Traffic* (2004)

Functional Rafts : A working hypothesis

pre-existing organization

induced rafts



Similarities in properties suggest similar mechanisms for formation for different lipid-anchored molecules

Unlikely to be a passive mechanism involving thermodynamic phase separation