A toolkit of fluorescent molecular probes for biomembrane research

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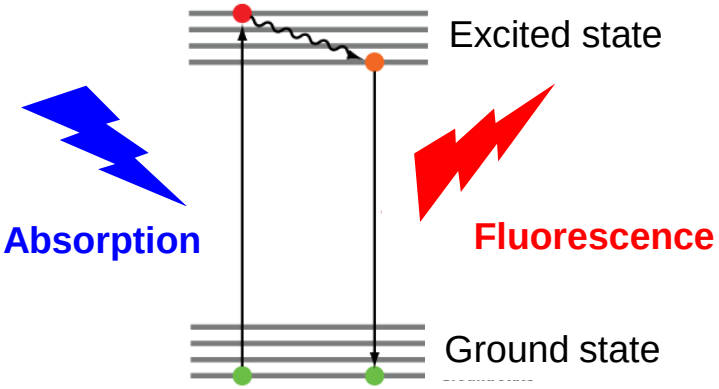
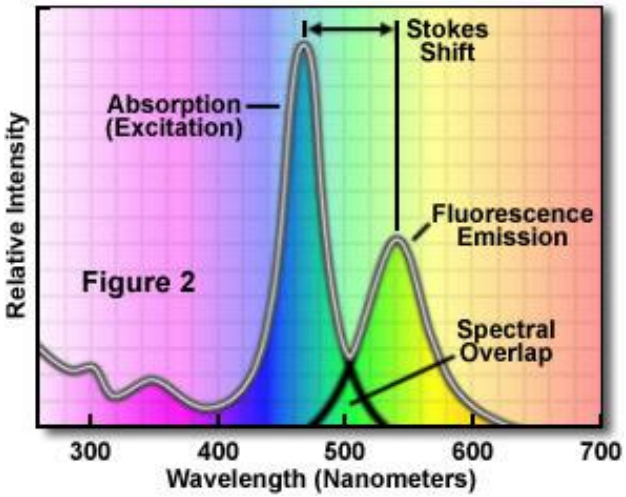
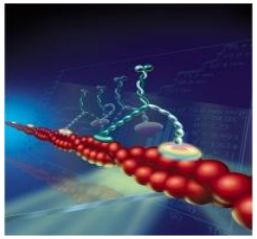
Why fluorescence?

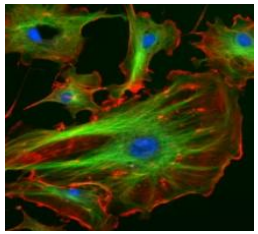


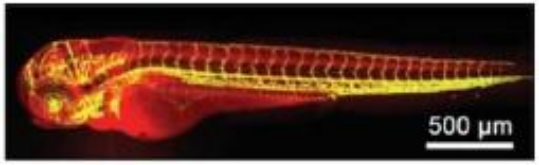


- **Sensitive:** up to single-molecule detection (10^{-6}-10^{-12} M).
- **Fast:** any time scale is available up to picoseconds.
- **Non-invasive:** minimum effect on the object of study
- **Specific:** using specially designed molecule tools: fluorescent probes.

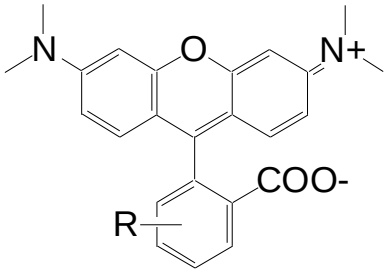
single molecule cell whole animal



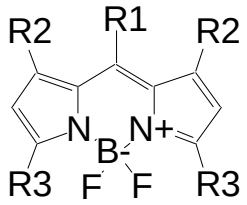




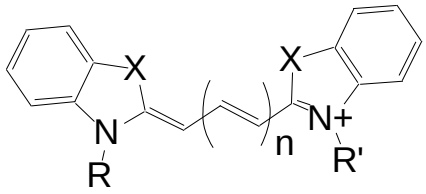
"Classical" fluorescent dyes



Rhodamine



BODIPY



Cyanine

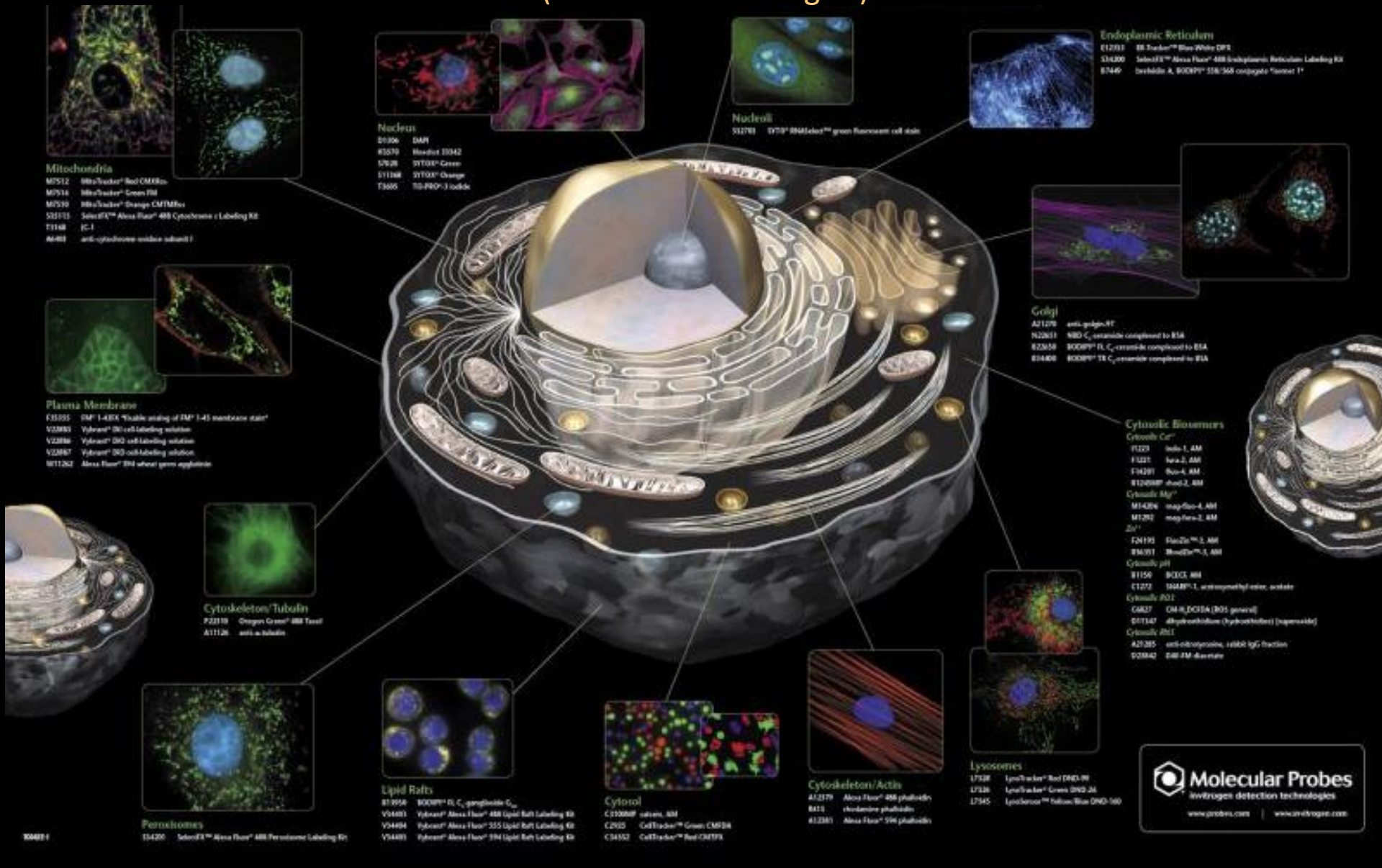
Cy3 (n=1)
Cy5 (n=2)

X = S, O, $C(CH_3)_2$

- Fluorescence of "classical" dyes is almost independent of the environment
- They are perfect markers, but they are not probes (unless sensor group is added).

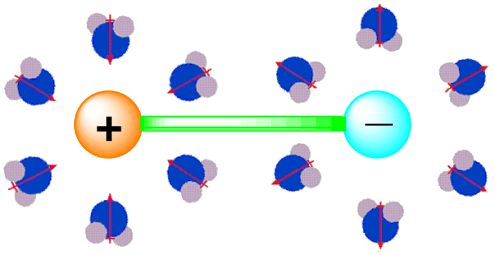
“The illuminated cell”: the successful project of Molecular Probes

(now Life Technologies)



Environment-sensitive fluorescent dyes

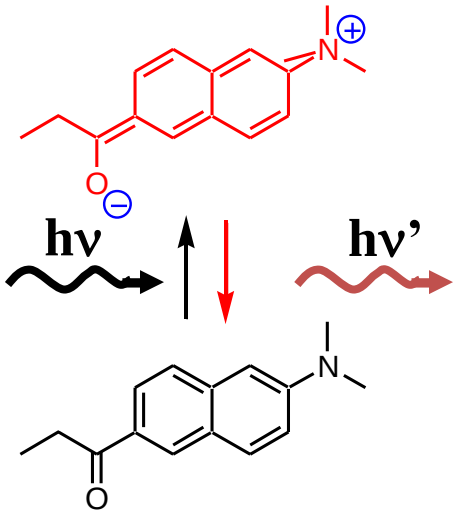
Water:
Polar



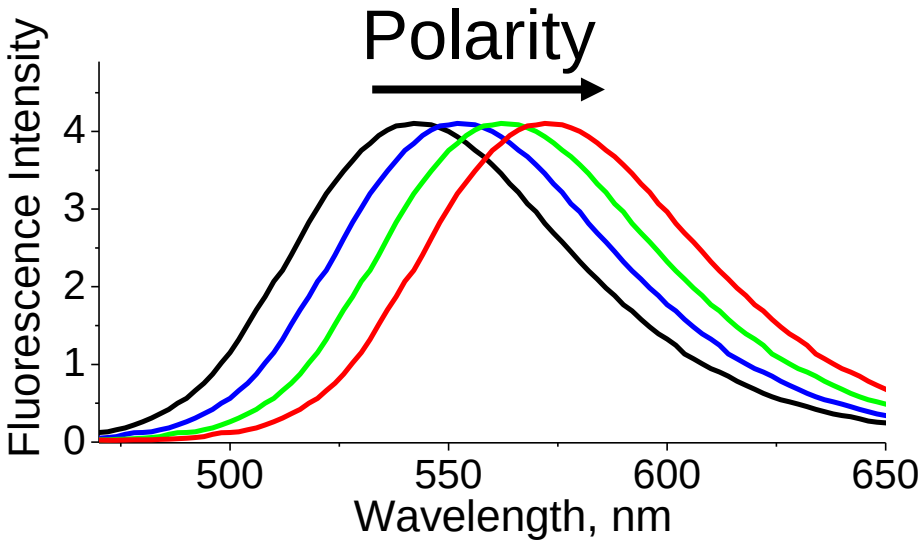
Strong solvation

Oil:
nonpolar

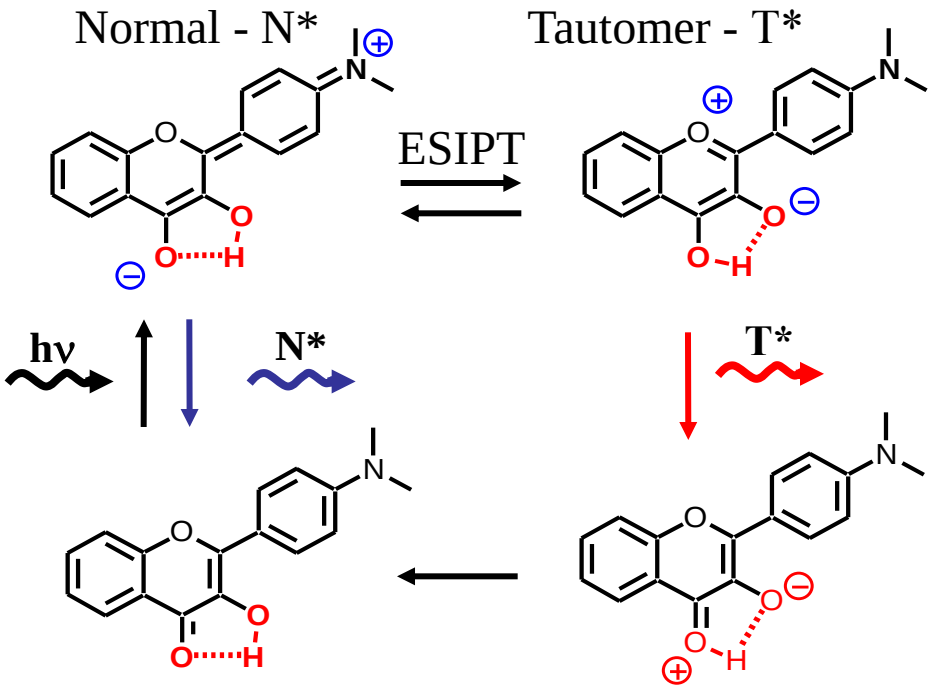
Poor solvation

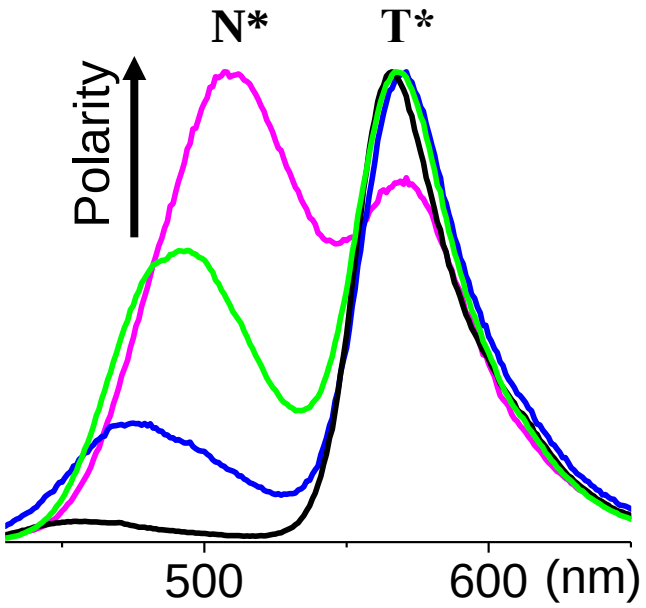


Prodan



3-Hydroxychromones (3HCs): Solvatochromic ESIPT dyes



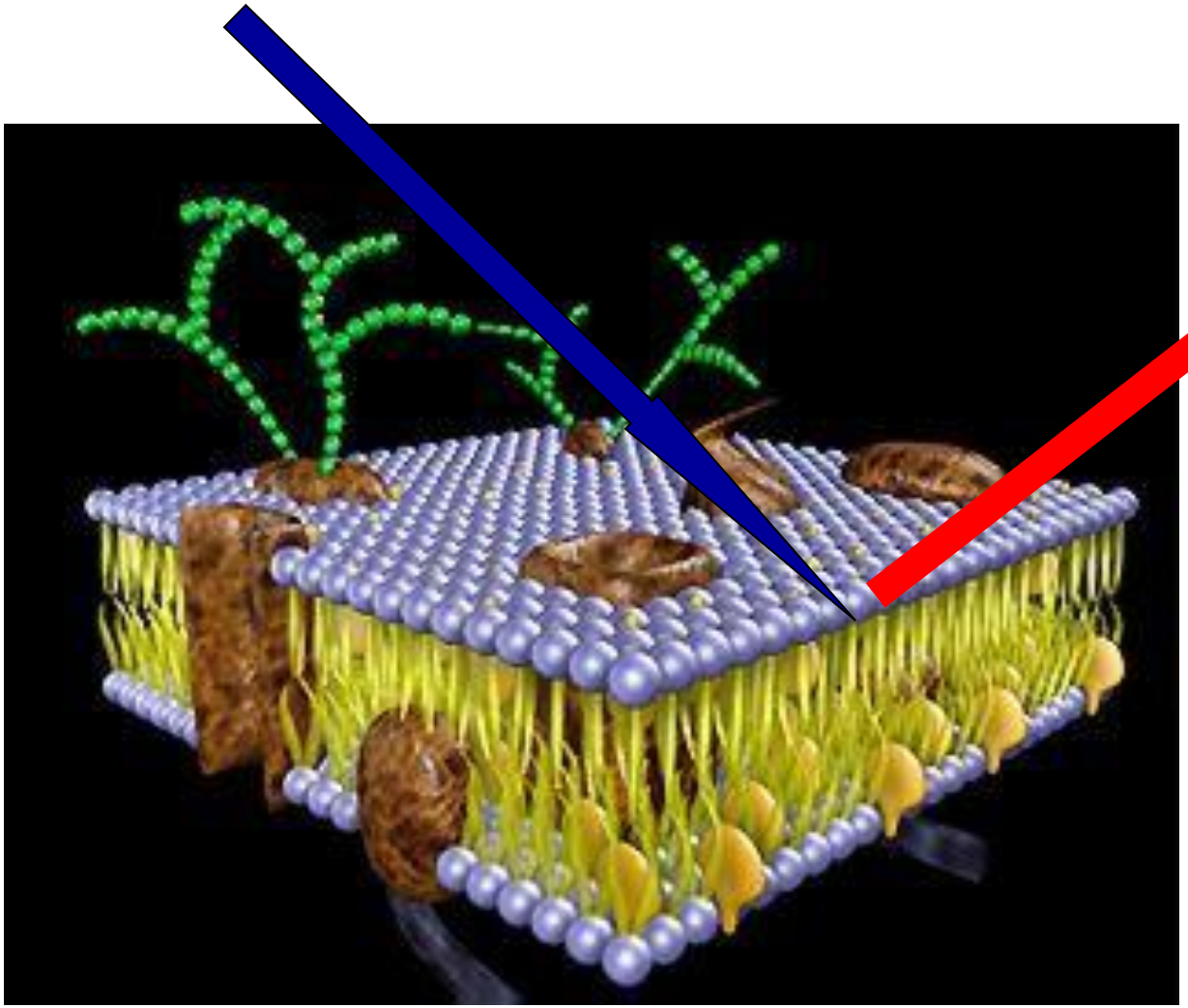


Advantages:

1) Satisfactory absorption properties: absorption ~400 nm (35,000 $M^{-1}cm^{-1}$).
2) Fluorescence quantum yield: 5-50 %
3) Extreme sensitivity of dual emission to environment.
4) Additional channels of spectroscopic information.

Sengupta & Kasha Chem. Phys. Lett. 1979, 68, 382.

Probes for membranes

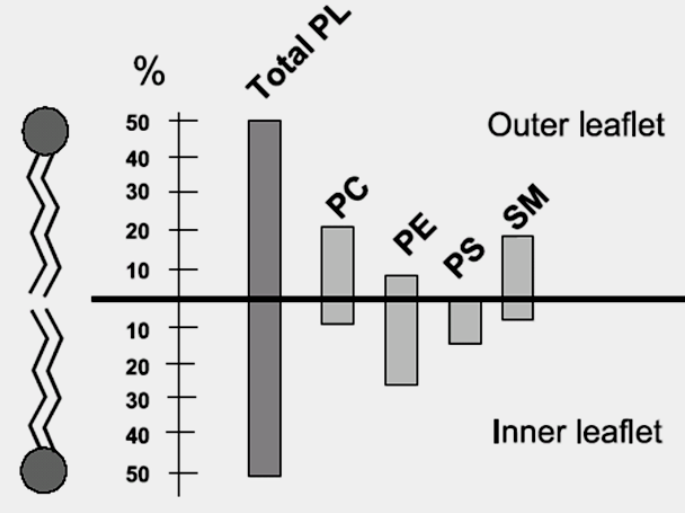


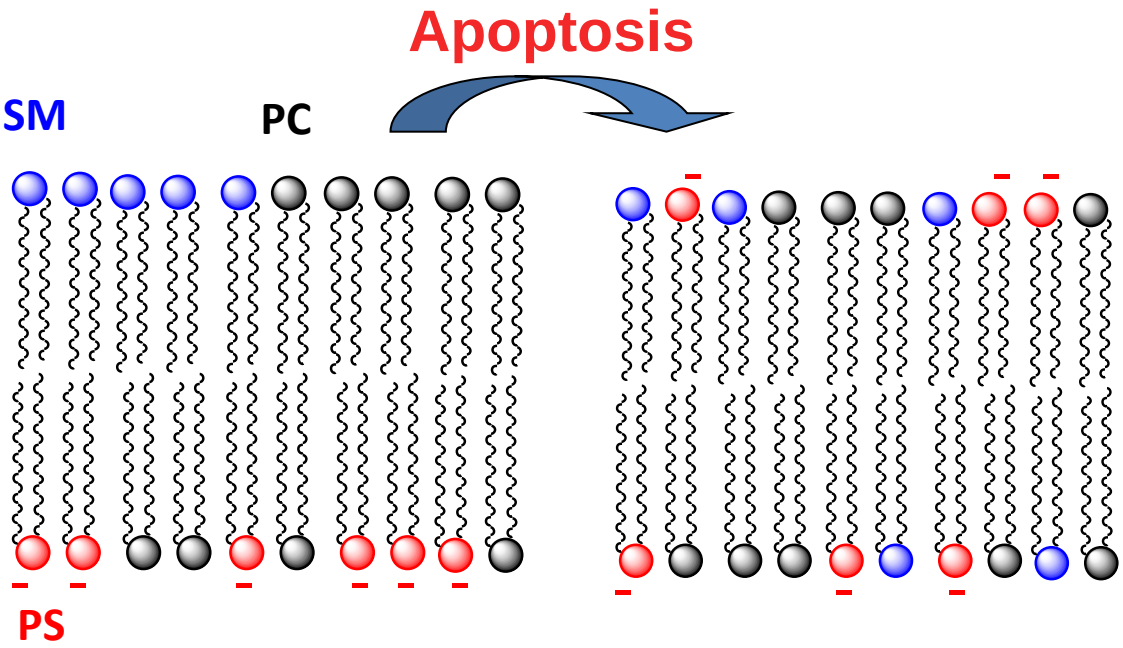
Fluorescence

Membrane structure,
lipid distribution,
& phase state

Cellular membrane and apoptosis

Transmembrane asymmetry in plasma membranes



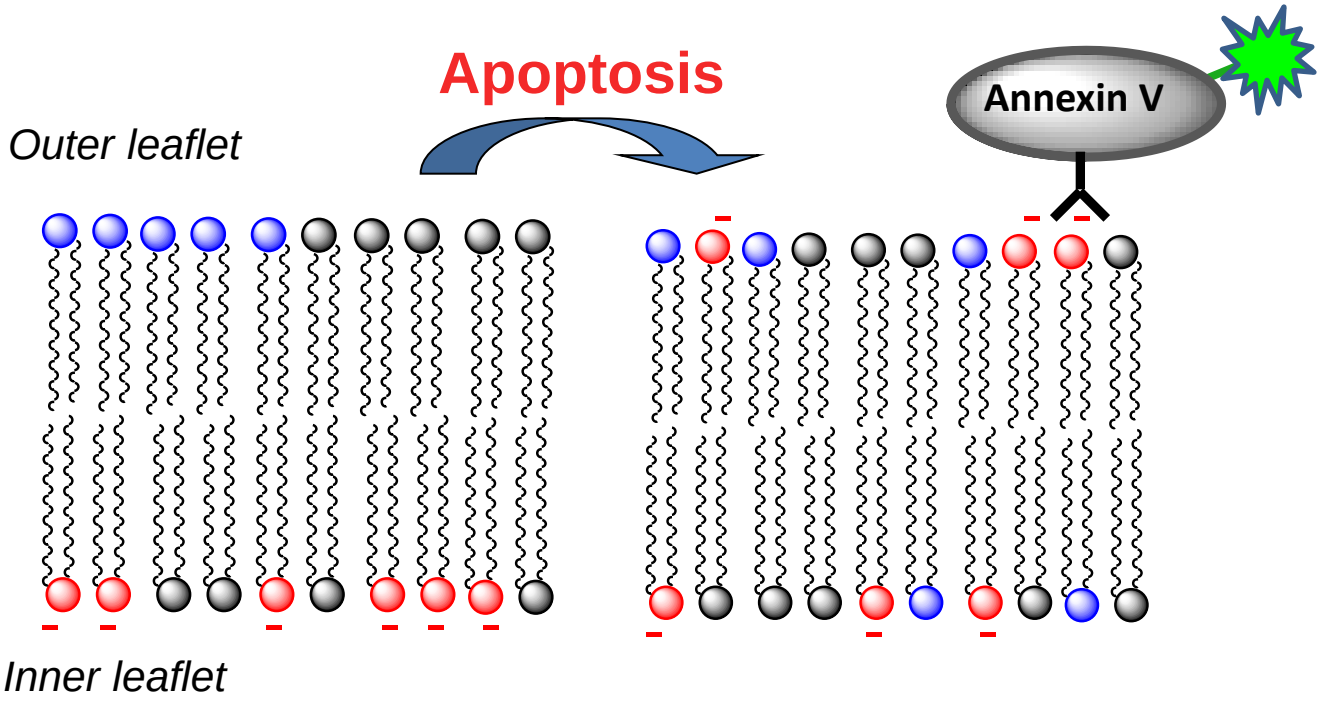


PC = phosphatidyl choline
PE = phosphatidyl ethanolamine
PS = phosphatidyl serine
SM = Sphyngomyelin

The asymmetry is lost on apoptosis
- ✓ Surface charge increases
- ✓ Lipid order decreased?

Zwaal, R. F., and A. J. Schroit, 1997, *Blood*. 89, 1121.

Cellular membrane and apoptosis

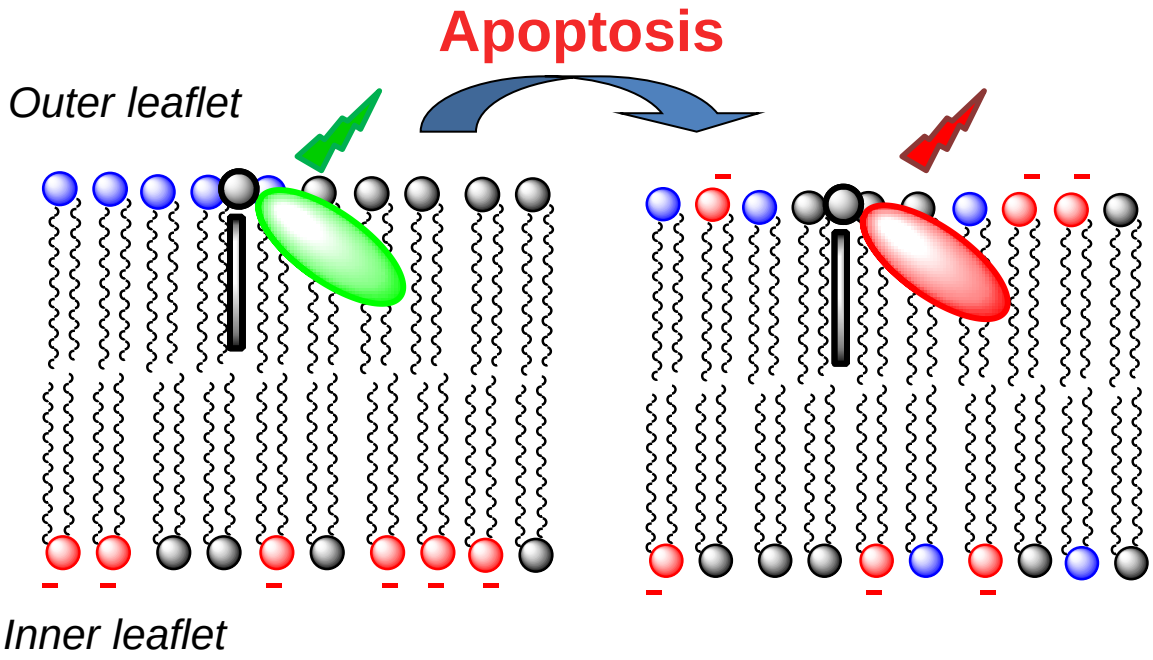


Van Engeland et al., ***Cytometry*** **1998.**

Some limitations of this assay:
- Ca2+-dependent
- Expensive
- Intensiometric

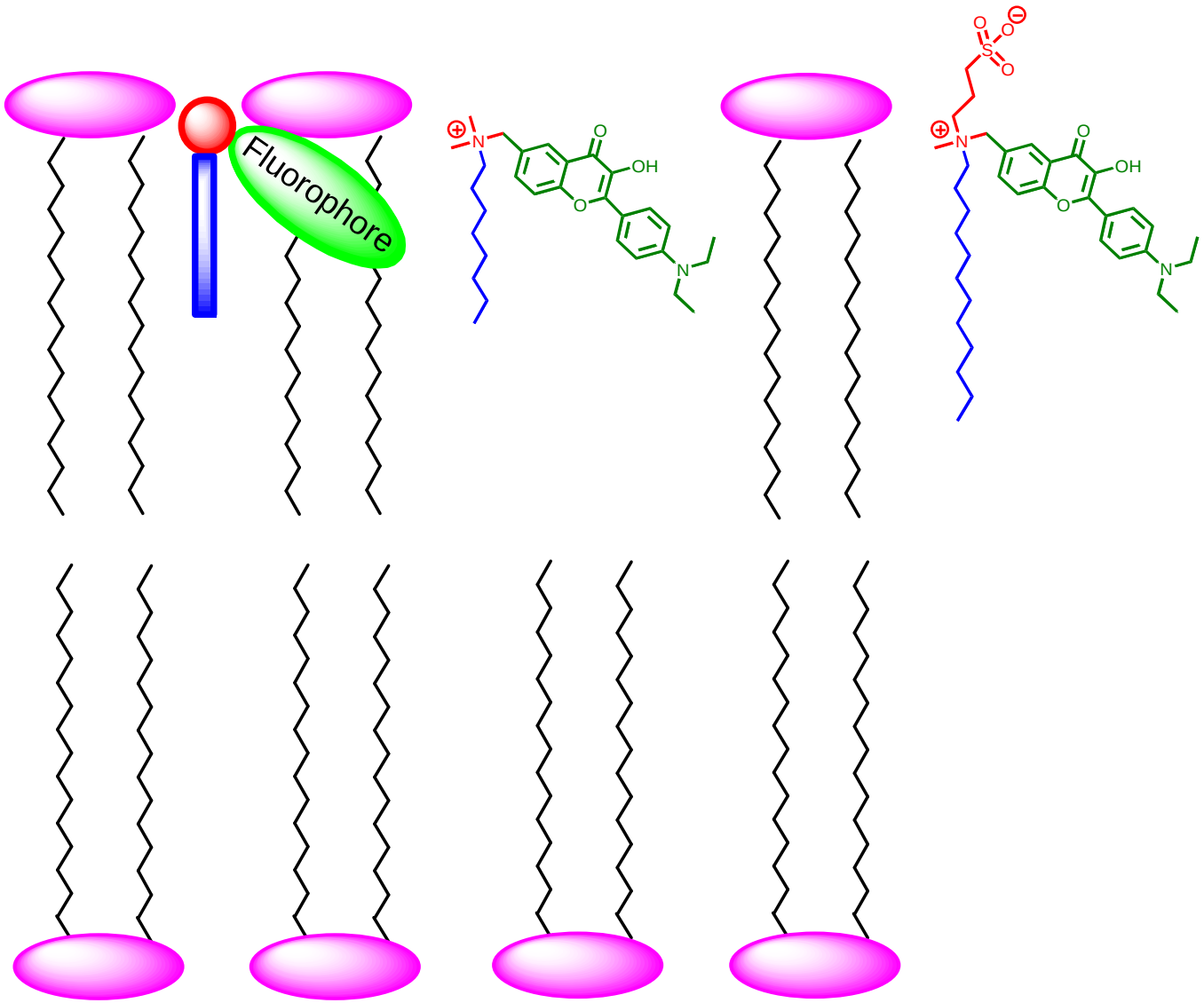
Alternative methods of apoptosis detection based on membrane changes

Cellular membrane and apoptosis



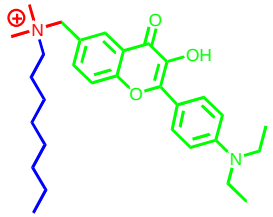
Dye should be sensitive to lipid composition:
surface charge and lipid order

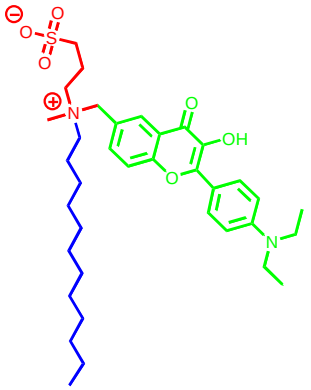
Localizing probe at the membrane



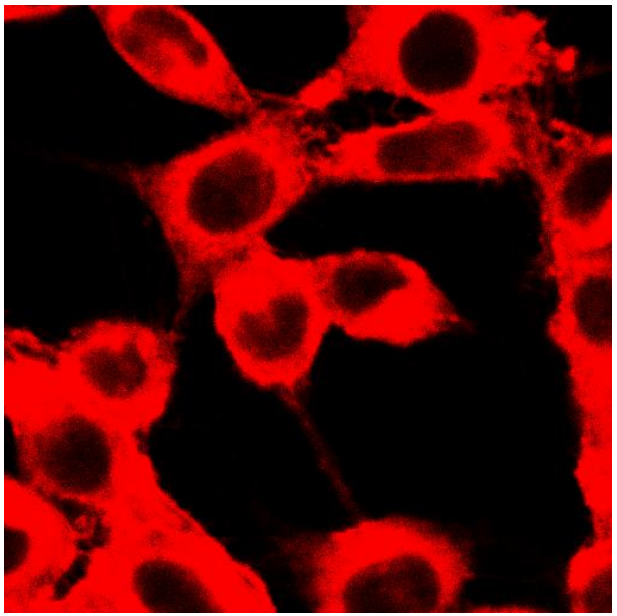
Shynkar et al, J. Am. Chem. Soc. 2007, 129, 2187

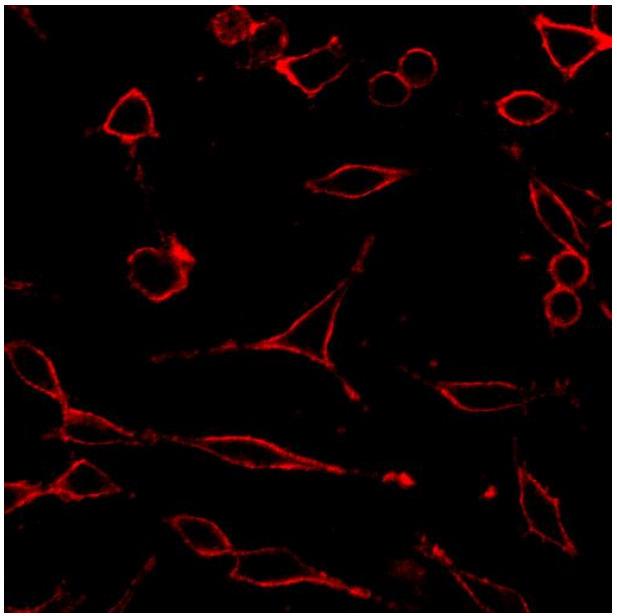
Design of the biomembrane probe





Internalize ☹ **Stains only membrane ☺**



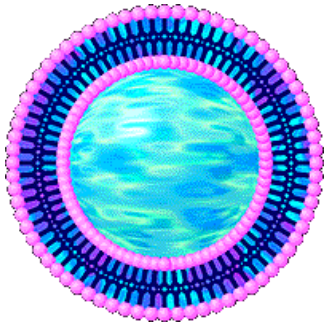


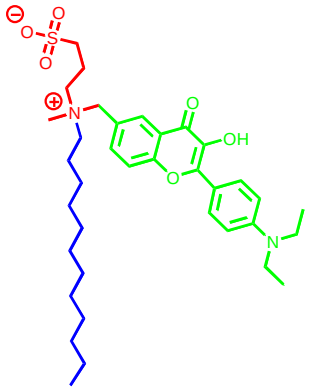
Confocal microscopy images

Fibroblast L 929 cell line stained with 1 μM of probes

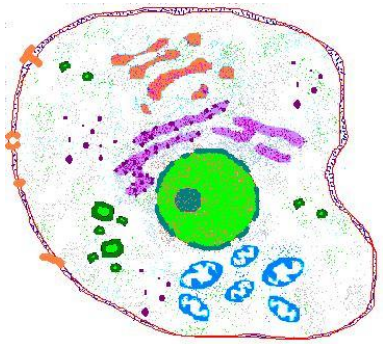
Response of F2N12S to surface charge in liposomes and to apoptosis in cells

In liposomes



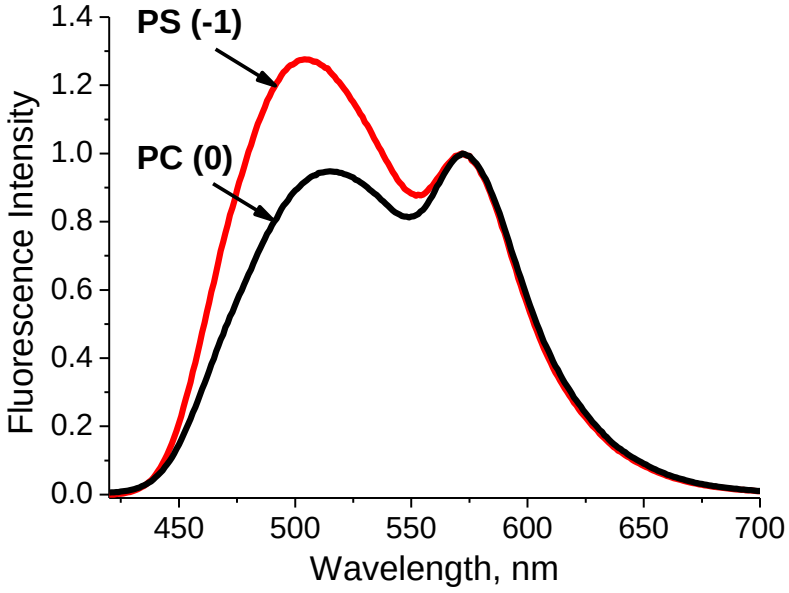


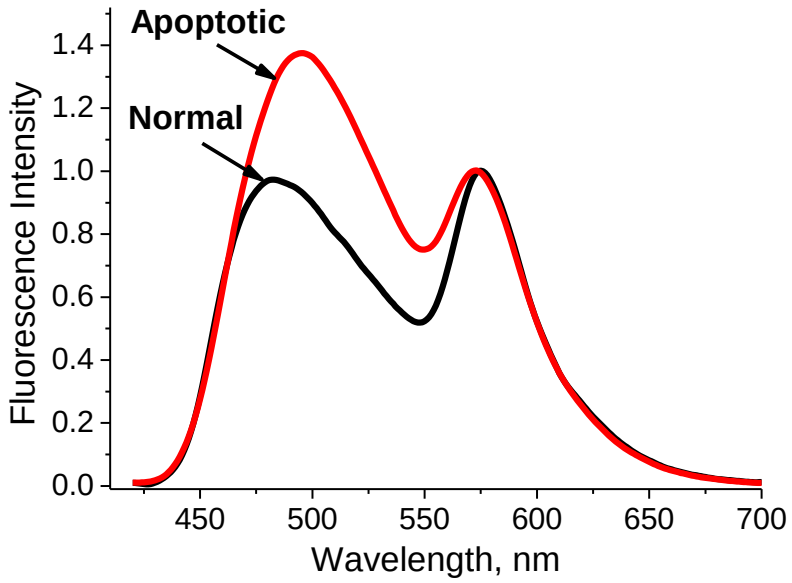
In cells



+actinomycin

Apoptosis



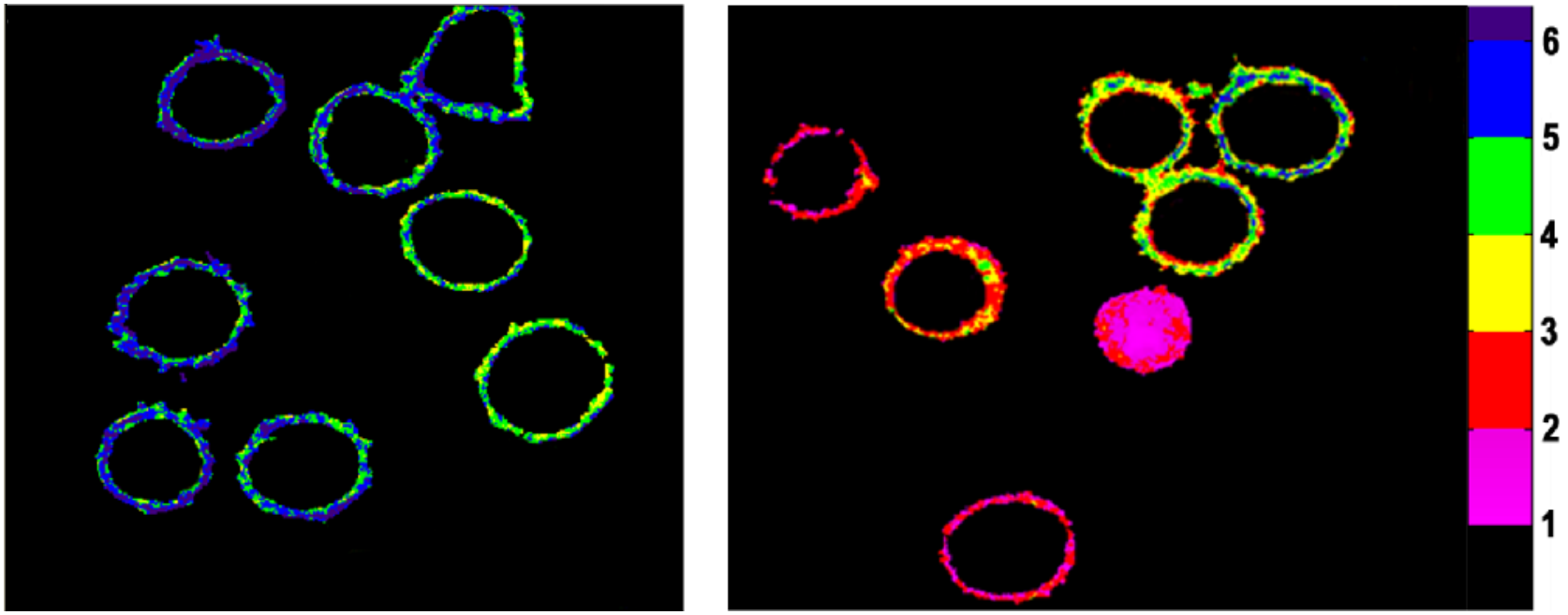


Shynkar et al, J. Am. Chem. Soc. 2007, 129, 2187.

Fluorescence microscopy imaging of apoptosis with probe F2N12S

Apoptosis

I_{Red} / I_{Blue}



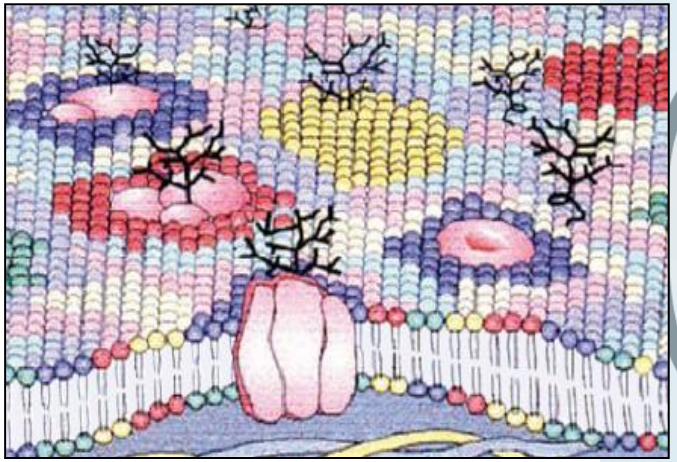
Non-treated

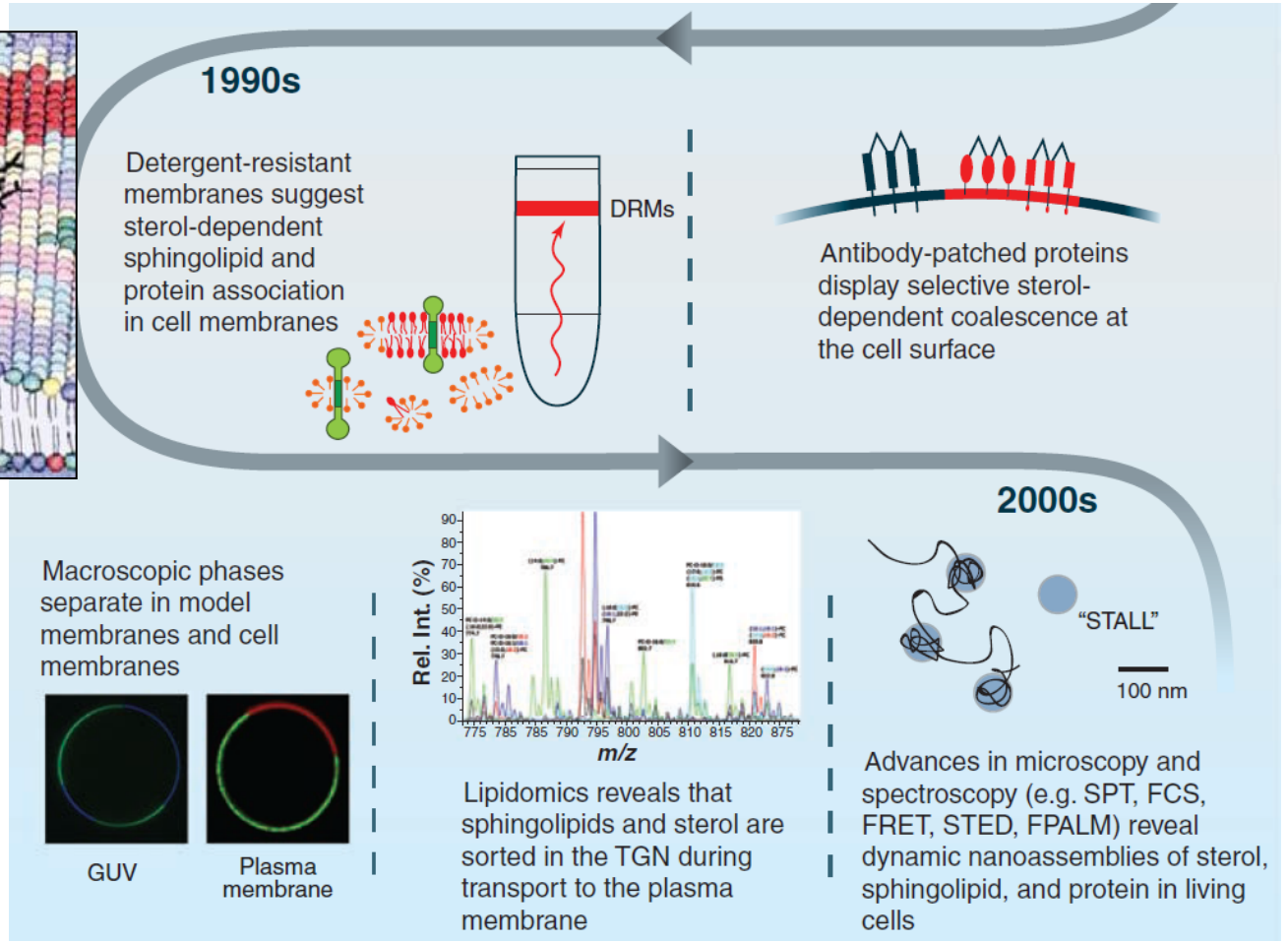
Treated with actinomycin for 18 h

CEM cell line stained with 1 μM of probes

Shynkar et al, J. Am. Chem. Soc. 2007, 129, 2187.

Hypothesis of "lipid rafts"

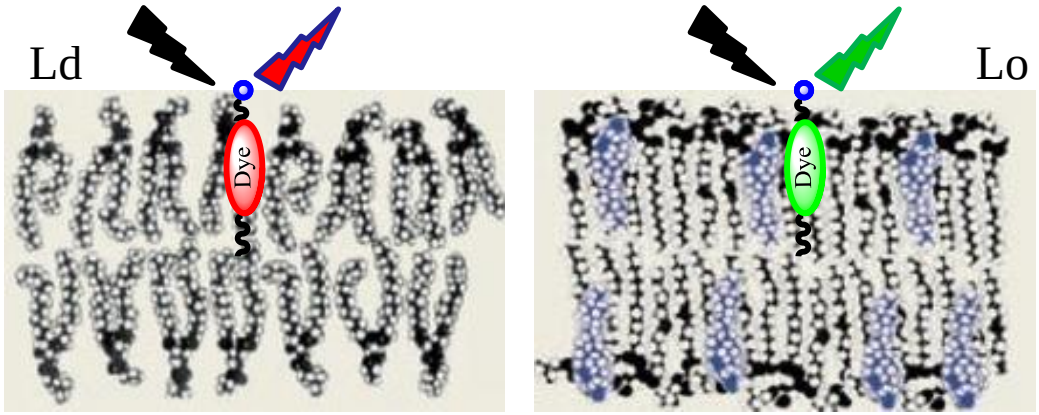




Lingwood, D., Simons, K. *Science* 2010, 327, 46

Fluorescent probes for lipid rafts: what is needed?

Phase-sensitivity

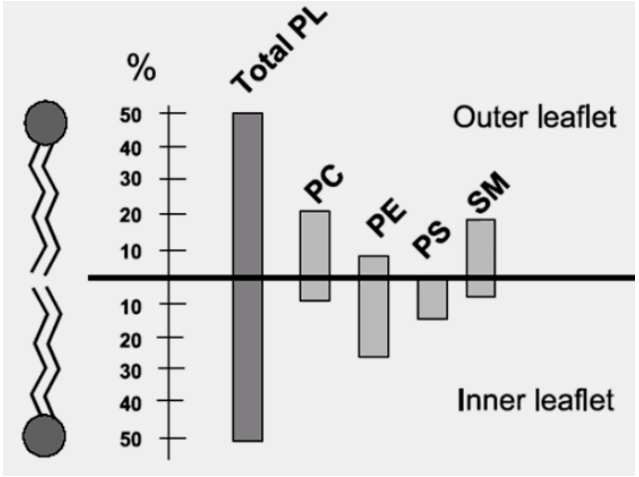


✓ Loosely packed
✓ Fast dynamics
✓ Hydrated
✓ Polar

✓ Tightly packed
✓ Slow dynamics
✓ Dehydrated
✓ Apolar

Use of environment-sensitive (solvatochromic) probes?

Leaflet-specificity



✓ Order in outer & inner leaflets is different

Specific staining of one leaflet

Klymchenko, A. S.; Kreder, R. *Chem. Biol.*, 2014, 21, 97.

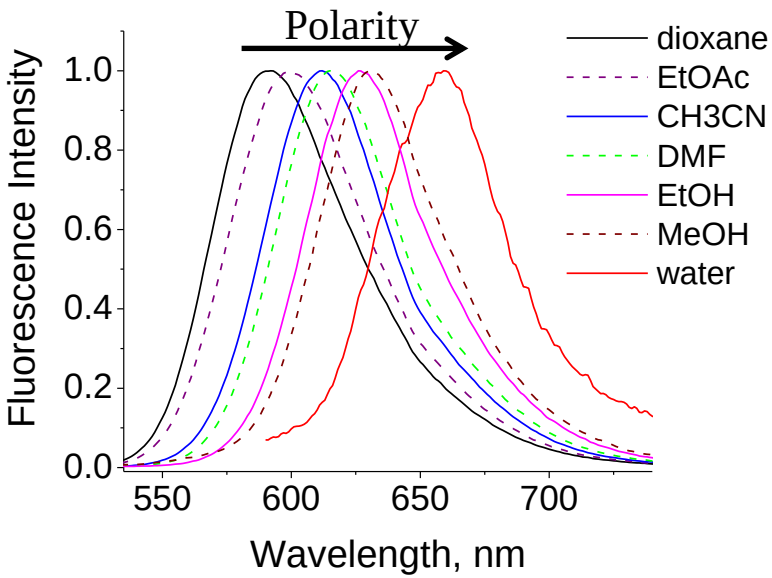
Nile Red-based probe

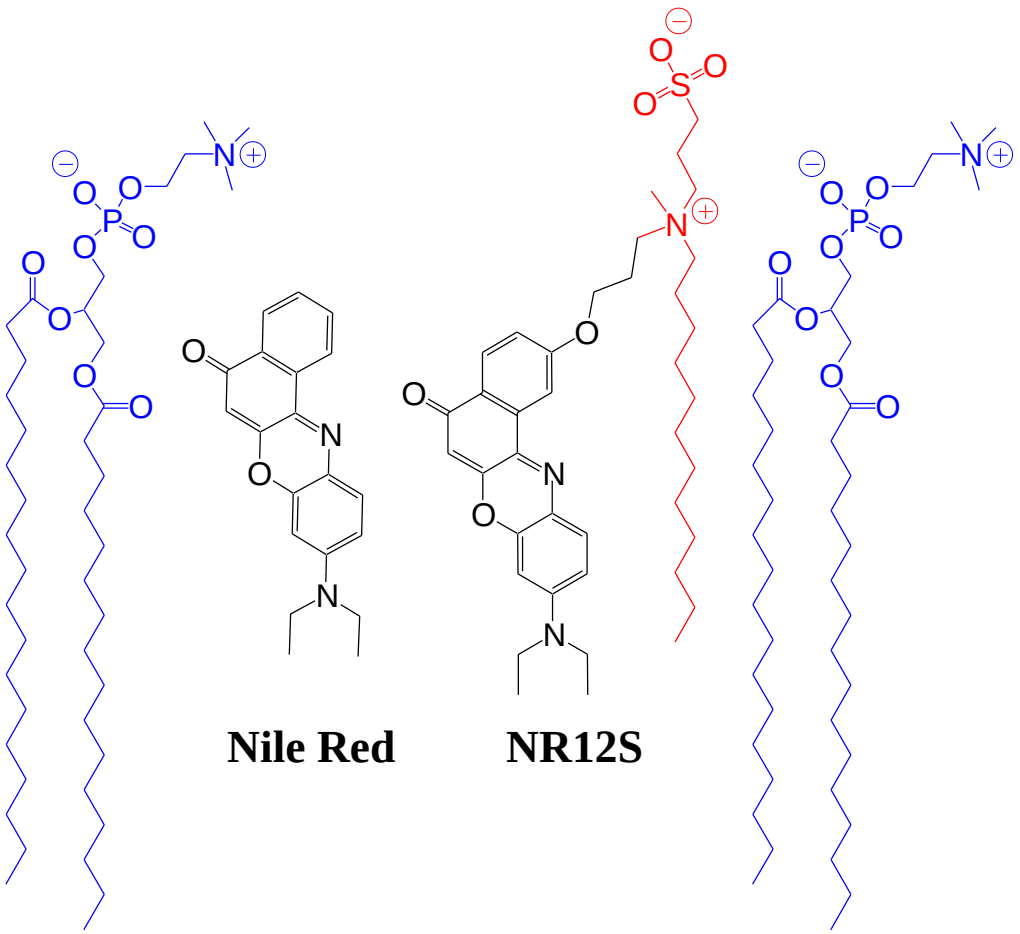
Key features of Nile Red:

1) Bright: ε = 40,000; QY~ 0.5.

2) Absorption max at 550 nm.

3) Environment-sensitive



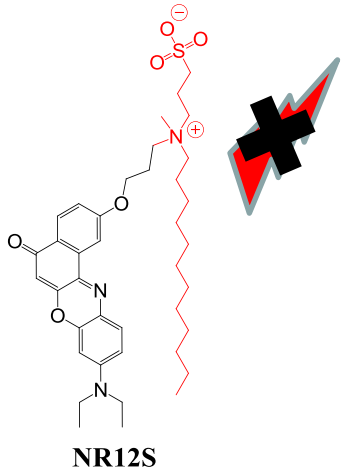


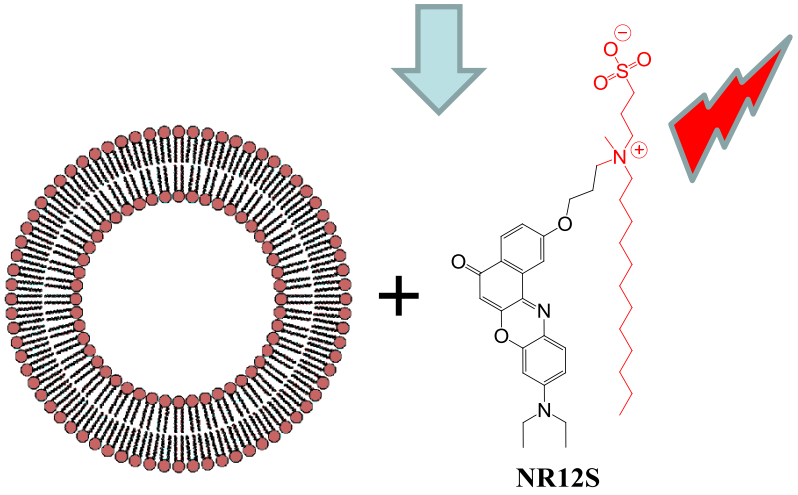
Nile Red **NR12S**

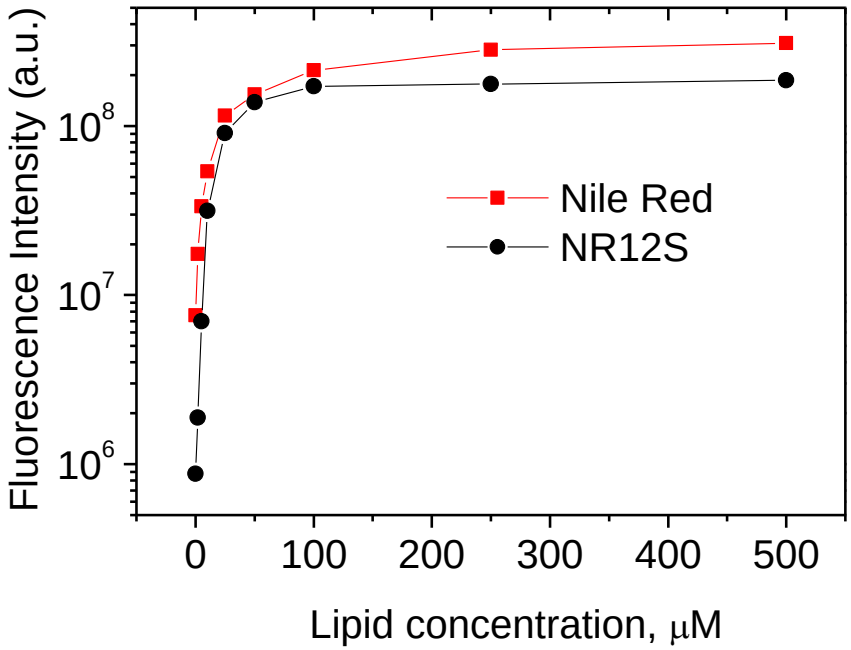
Kucherak et al., J. Am. Chem Soc. 2010, 132, 4907.

Fluorescence of NR12S "turns on" after binding to membranes

In water

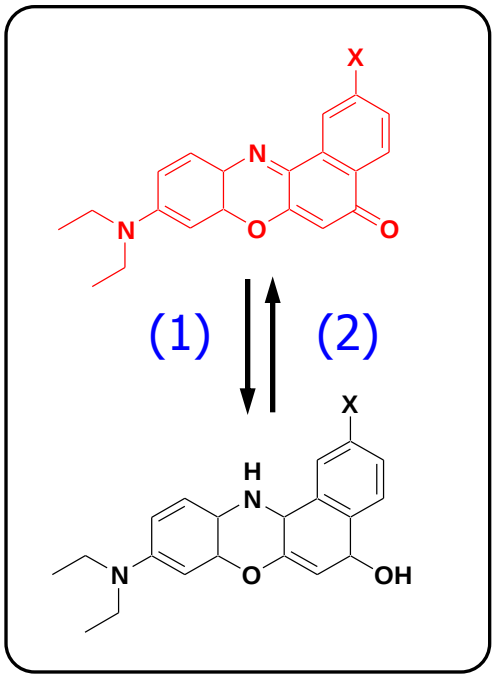






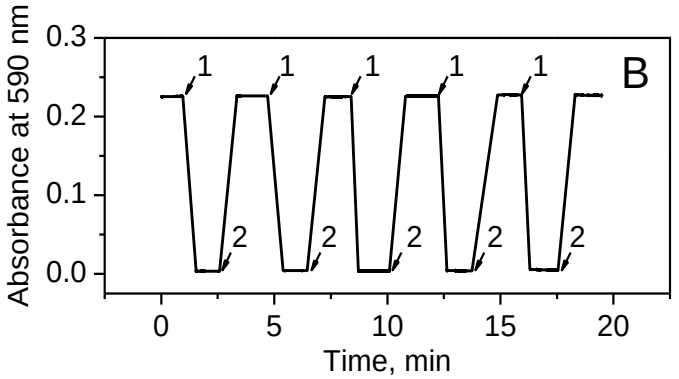
ON/OFF switching

state ON
Fluorescent
Red color



state OFF
non fluorescent
Colorless

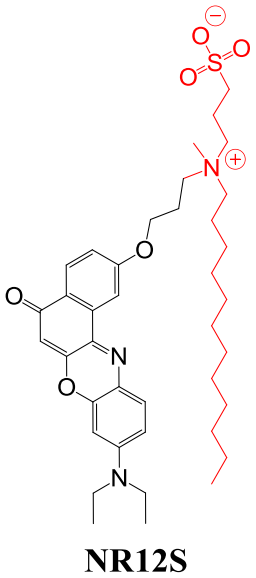
Switching in absorption of Nile Red

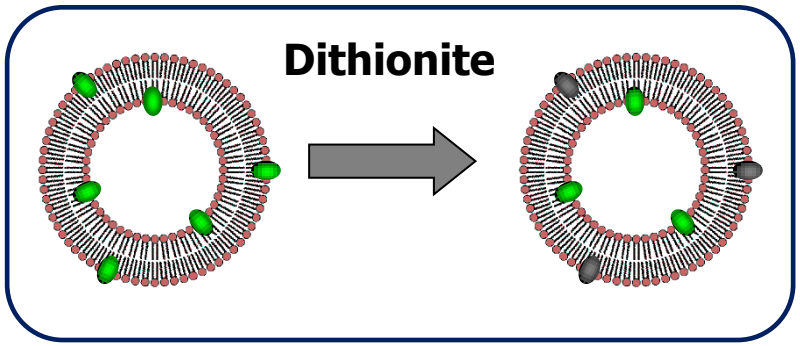


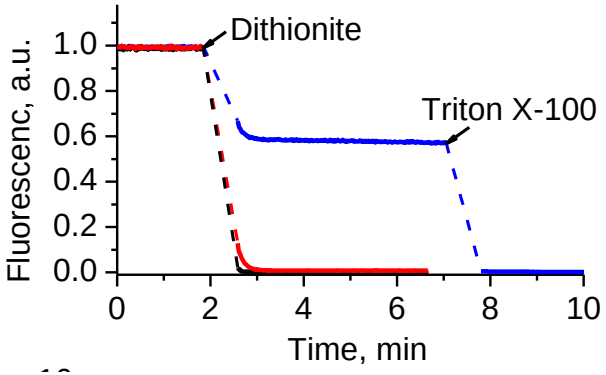
(1) addition of dithionite
(2) air bubbling

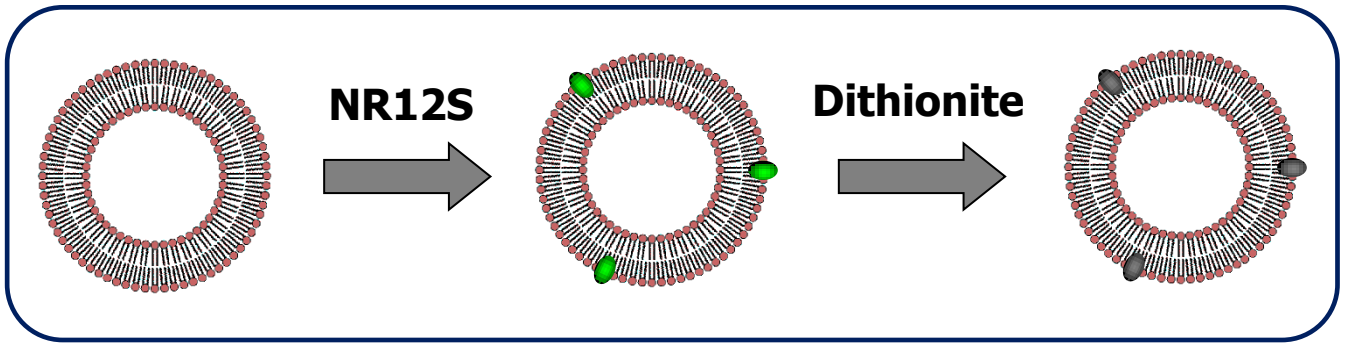
NR12S can be switched ON-OFF by dithionite and air

Selectivity to outer leaflet



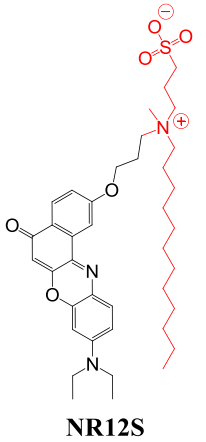


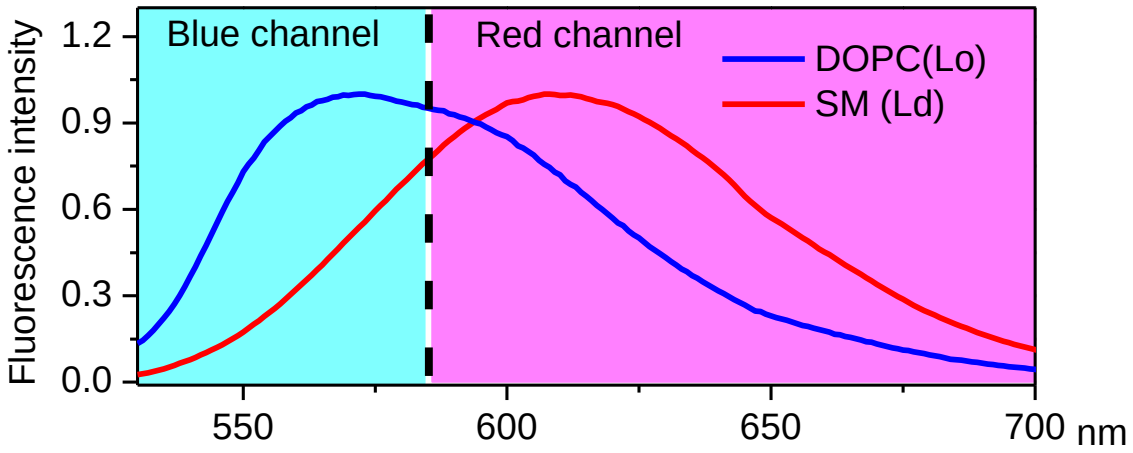




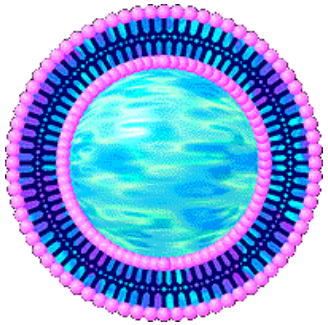
NR12S binds selectively outer membrane leaflet without detectable flip-flop

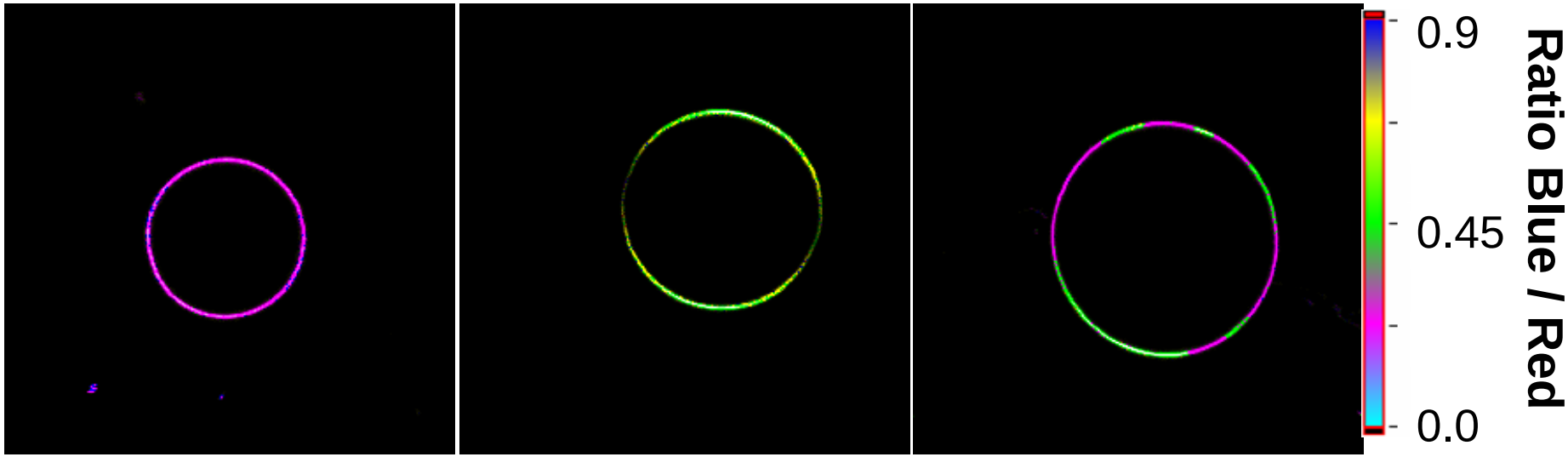
Probing and imaging membrane phases in model membranes by NR12S





In liposomes

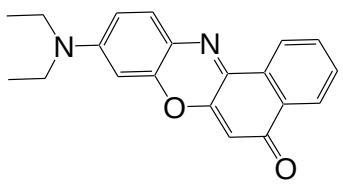




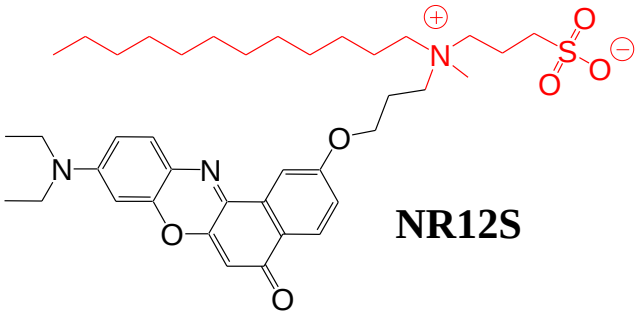
Ld phase **Lo phase** **Lo/Ld mixture**

NR12S provides good color contrast of different phase domains

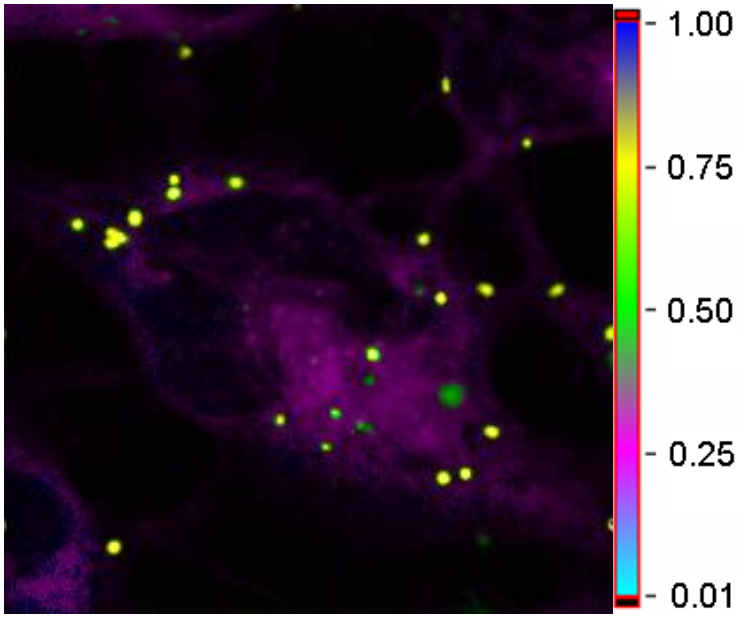
Selective staining of cell plasma membranes



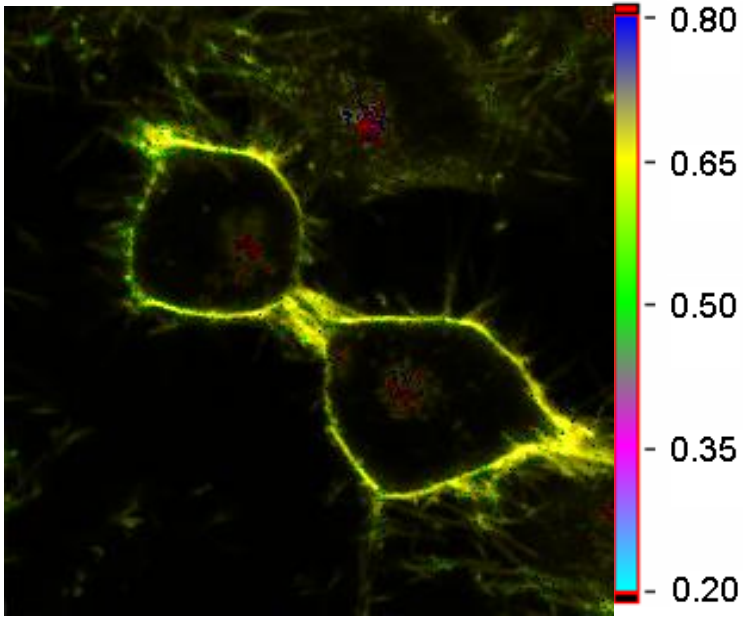
Nile Red



NR12S



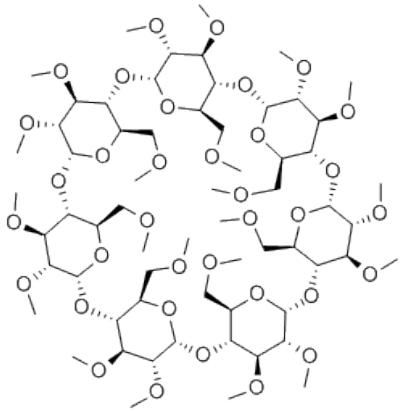
All cell + lipid droplets



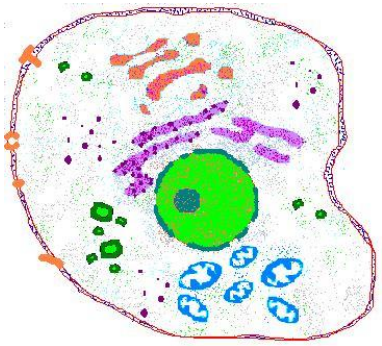
Cell membranes only!

NR12S binds exclusively cell plasma membranes

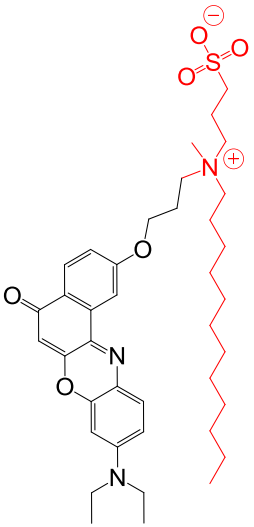
Color-Imaging of cholesterol content

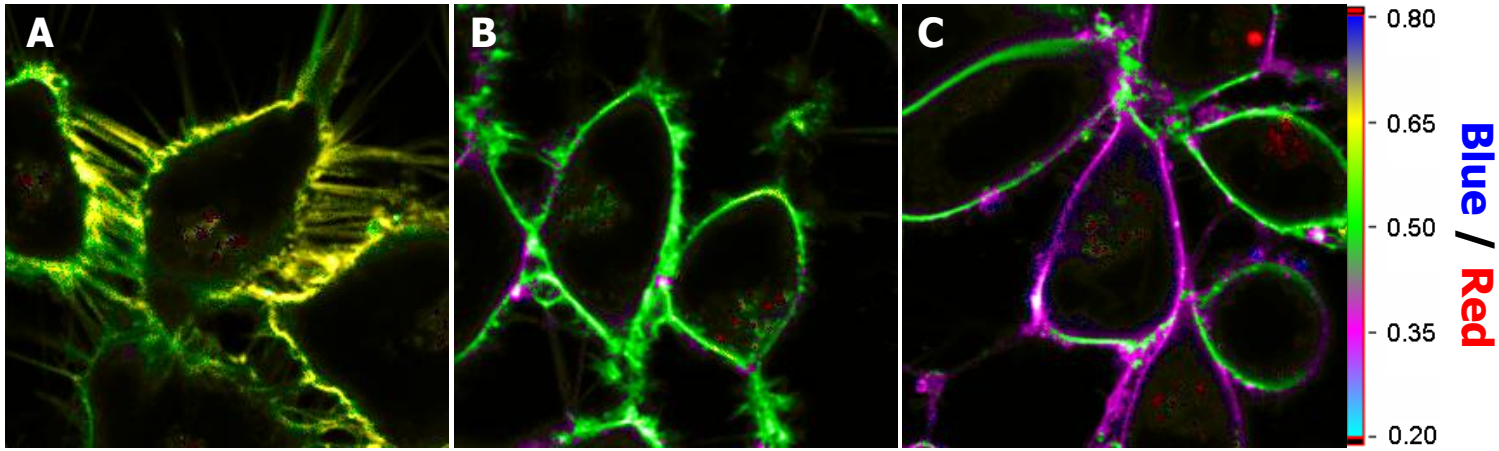


+



Cholesterol extraction by Methyl-ß-cyclodextrin





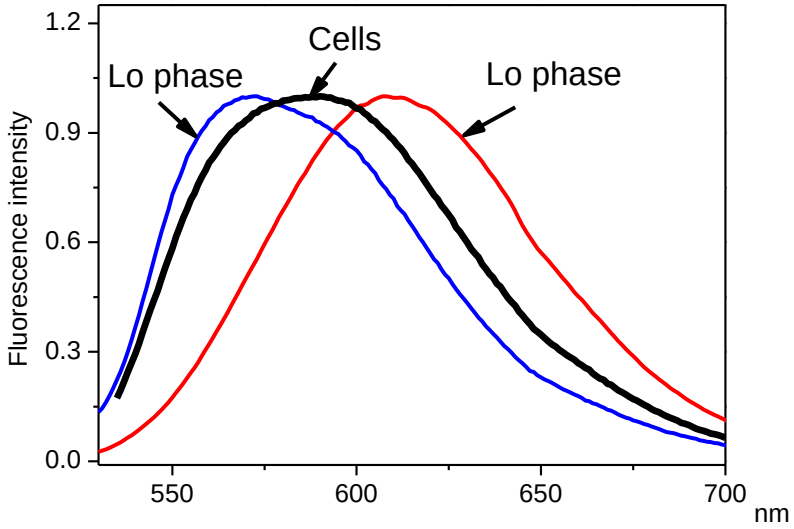
Control **30 min** **120 min**

Kucherak et al., JACS 2010

NR12S is sensitive to cholesterol content in the membrane

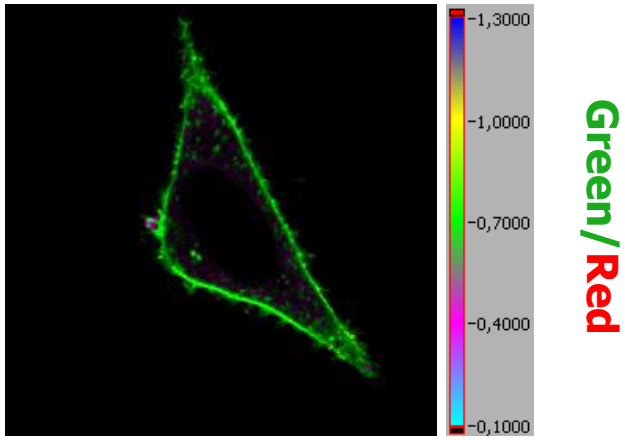
Where are the Rafts in Biomembranes?

LUVs and cells



- Intermediate spectrum Lo-Ld

Intact cells



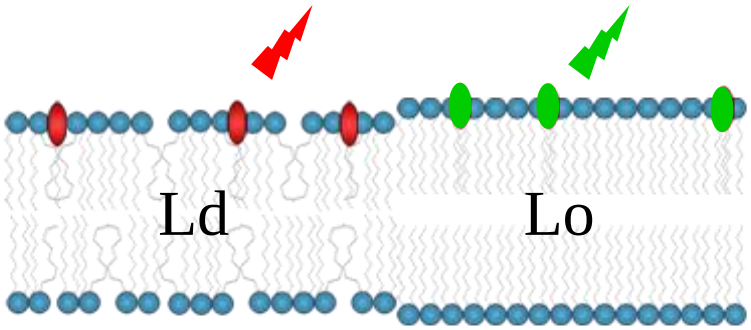
Green/Red

- But impossible to detect rafts on intact cells
- Limited temporal and spatial resolution of optical microscopy

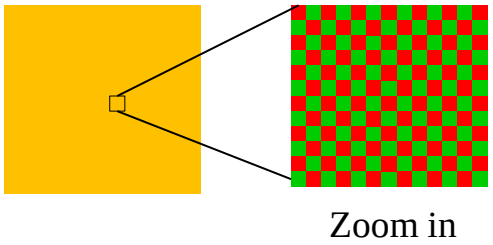
Darwich et al., BBA 2012

Phase-specific Nile Red probe

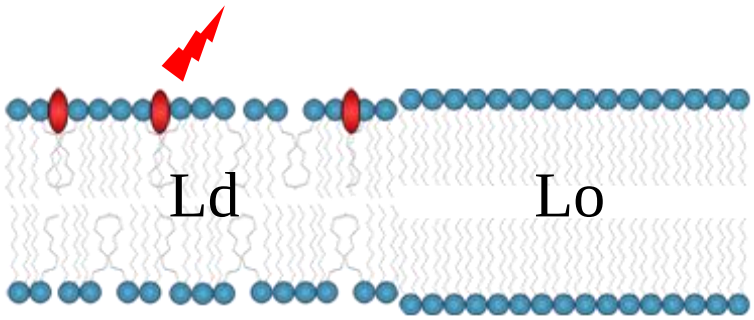
- NR12S binds both phases



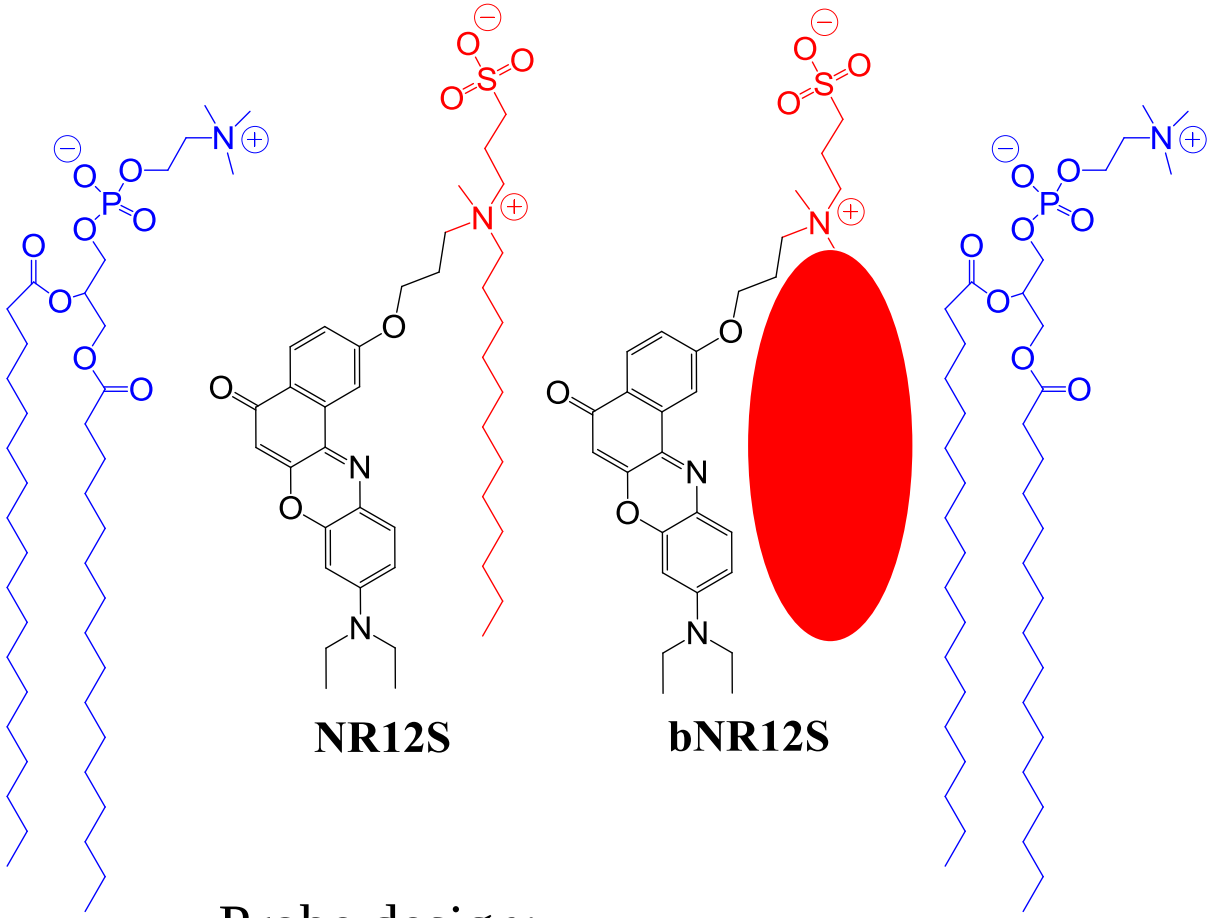
Colors mix up:



- "Bulky" NR12S analogue binding specifically to Ld phase



Design of the probes

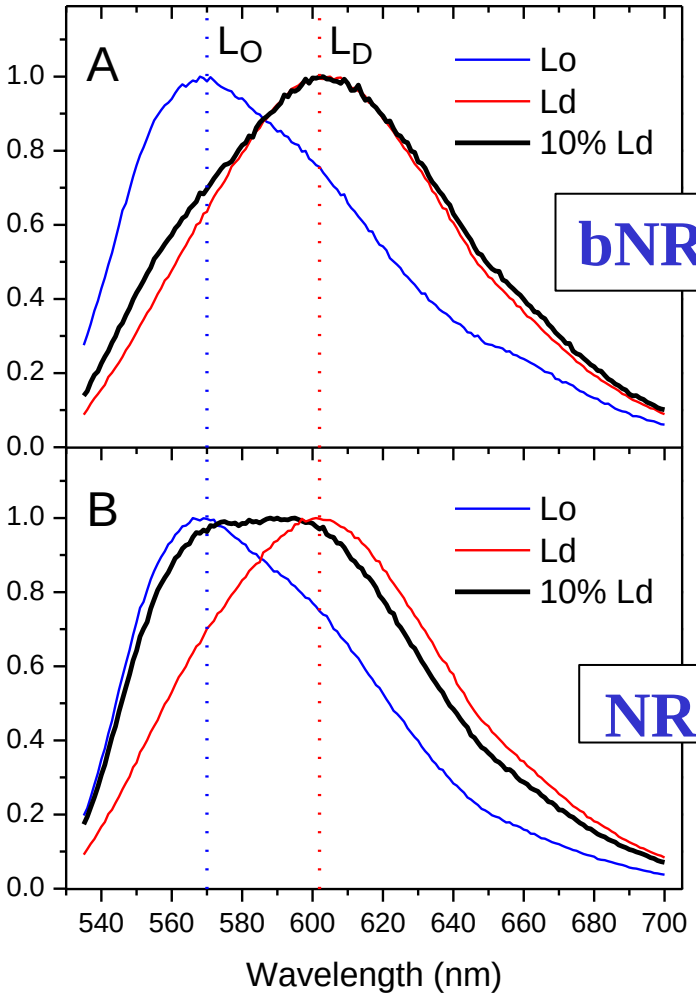


NR12S **bNR12S**

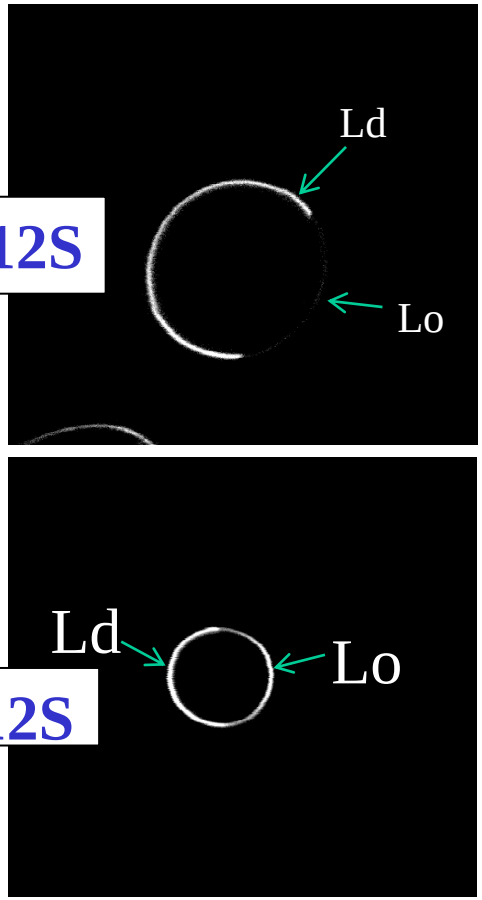
- Probe design:
  - Analogue of NR12S
  - A bulky alkyl chain

Validation in model membranes and cells

100-nm vesicles

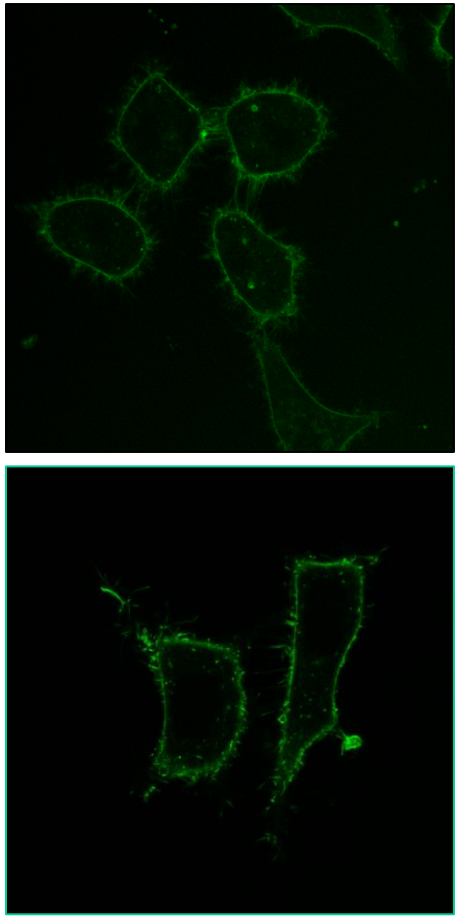


Giant vesicles



Lo – Sphyngomyelin/Cholesterol,
Ld – DOPC/Cholesterol

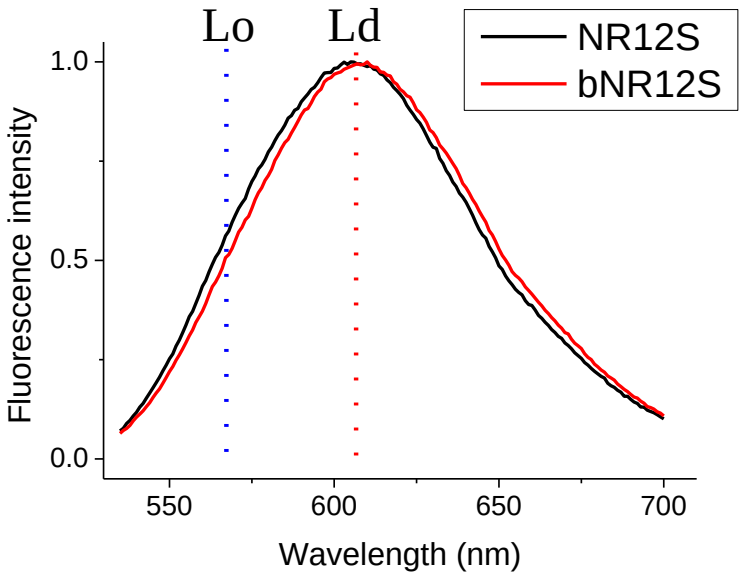
Cells



Hela cells stained with 0.4μM of probes

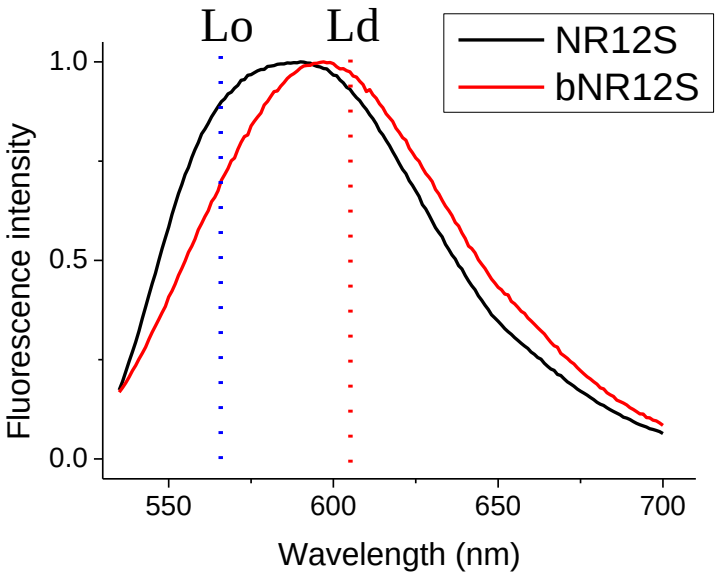
Comparison of NR12S and bNR12S in liposomes and HeLa cells

LUVs, Ld phase (DOPC)



Same spectra:
Homogeneous Ld phase

HeLa cells

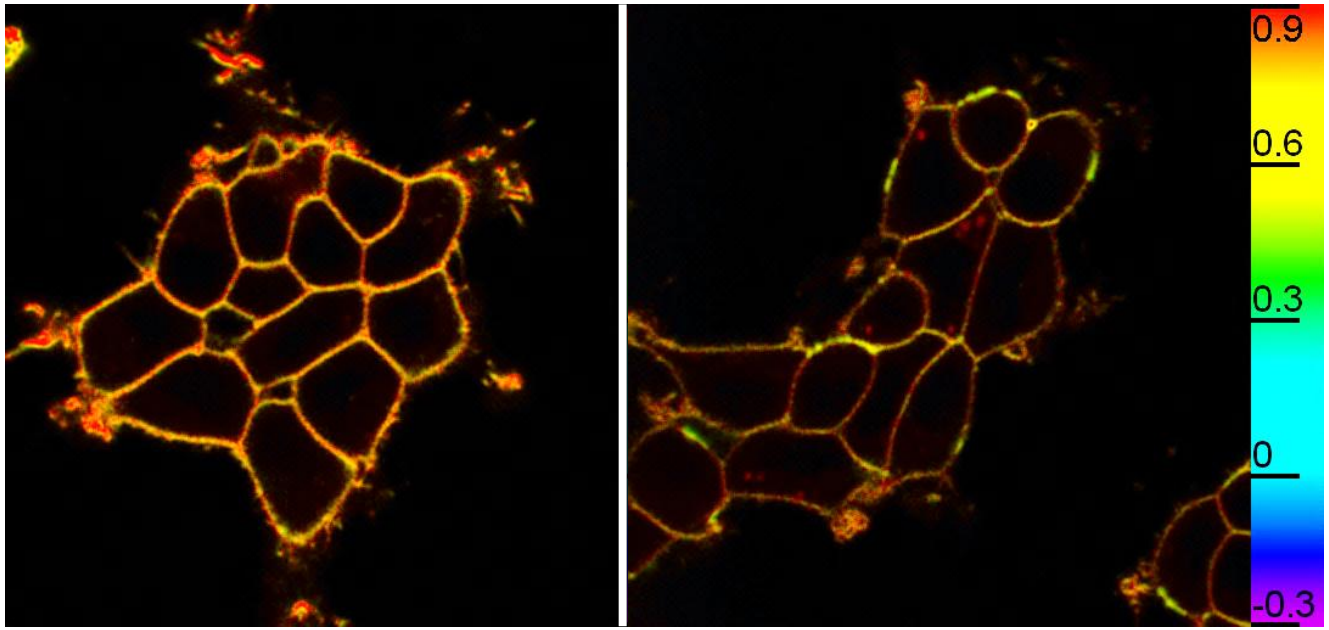


Different spectra:
bNR12S is excluded from Lo phase
NR12S partitions between Lo&Ld phases?
Coexistence of Ld&Lo phases!?

Direct observation of lipid rafts using phase-specific Nile Red probe

HEK293 cells stained with

NR12S bNR12S **Green/Red**



Homogeneous: poor specificity to phases

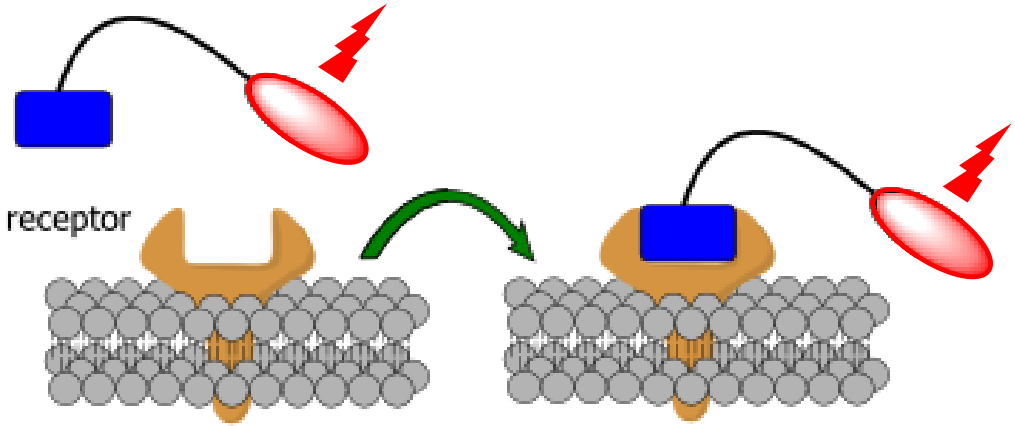
Domains of disordered phase can be distinguished by intensity and color!

Detection of membrane receptors

Classical Approach

Unbound ligand
Is fluorescent

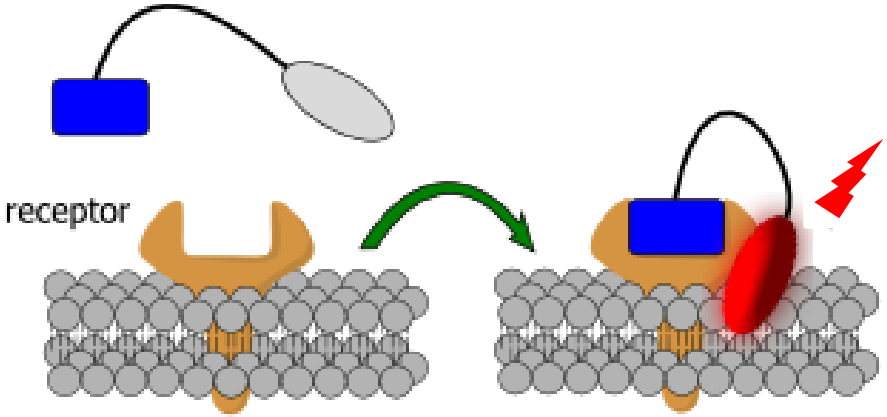
"Washing" is needed



"Turn-ON" Approach

Unbound ligand
is NOT fluorescent

No "Washing" needed



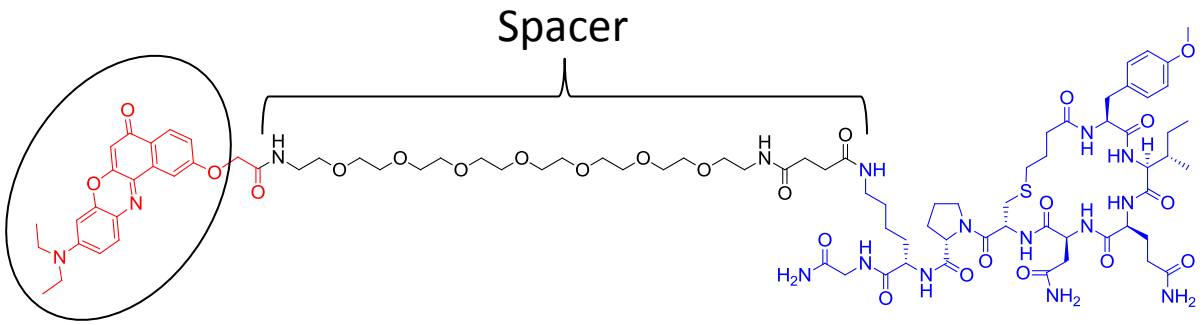
Problem:
Non-specific
interactions

Collaboration with D. Bonnet, M. Hibert, J. Karpenko, UMR 7200

« Turn – on » ligands for G protein-coupled receptors (oxytocin)

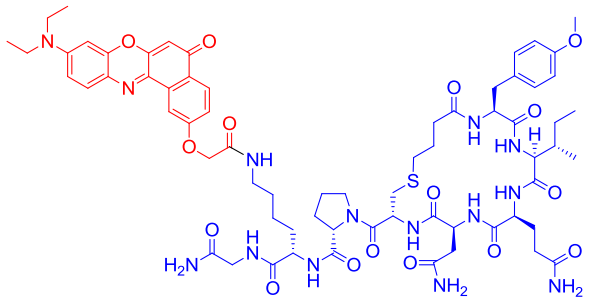
GPCRs is the target of ~ 40% of drugs on the market.

Nile Red
Turn-on dye:
Water – "OFF"
Oil – "ON"



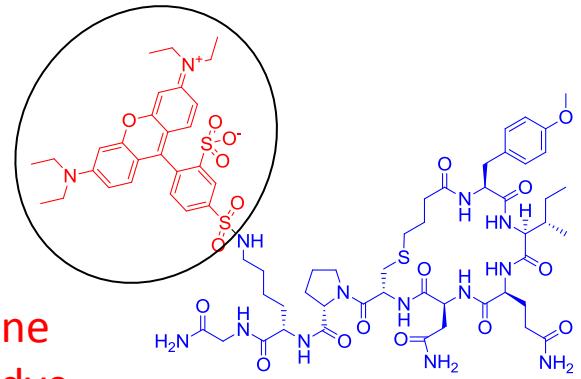
Carbetocin

NR-PEG-CBT



NR-CBT

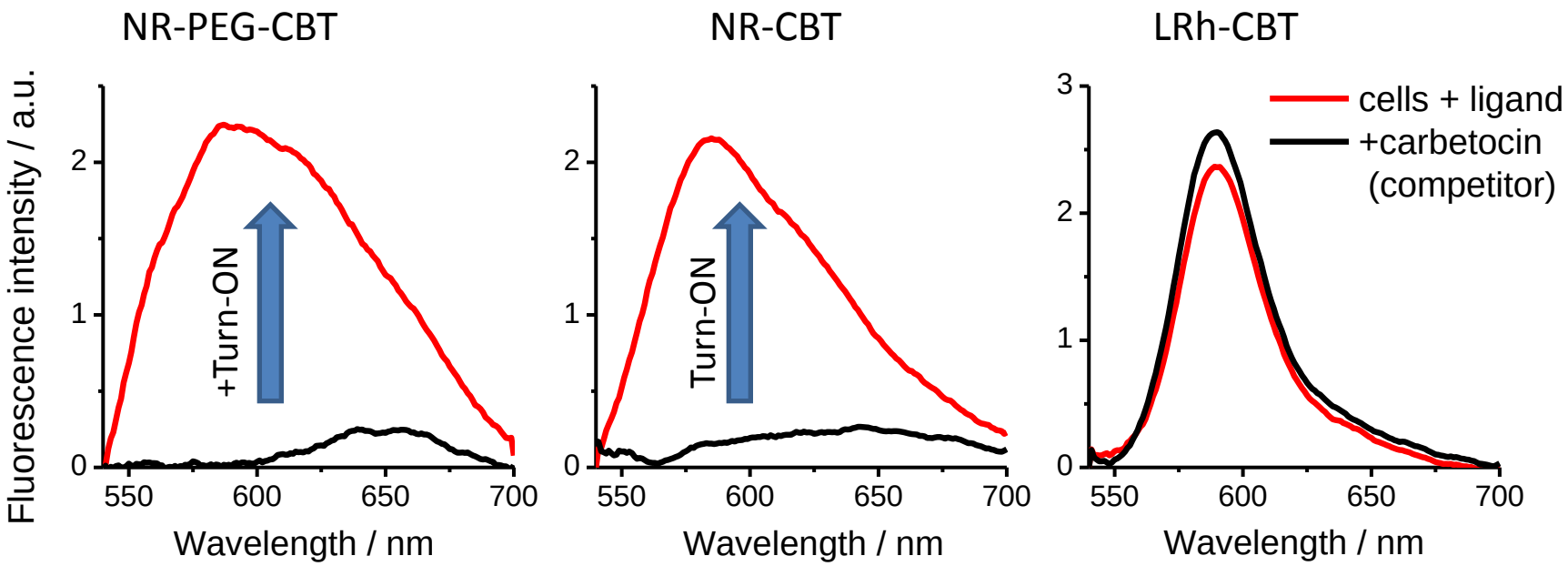
Rhodamine
Classical dye
Always "ON"



LRh-CBT

Collaboration with D. Bonnet, M. Hibert, J. Karpenko, UMR 7200

Turn-on vs classical ligands in suspensions of HEK 293 cells

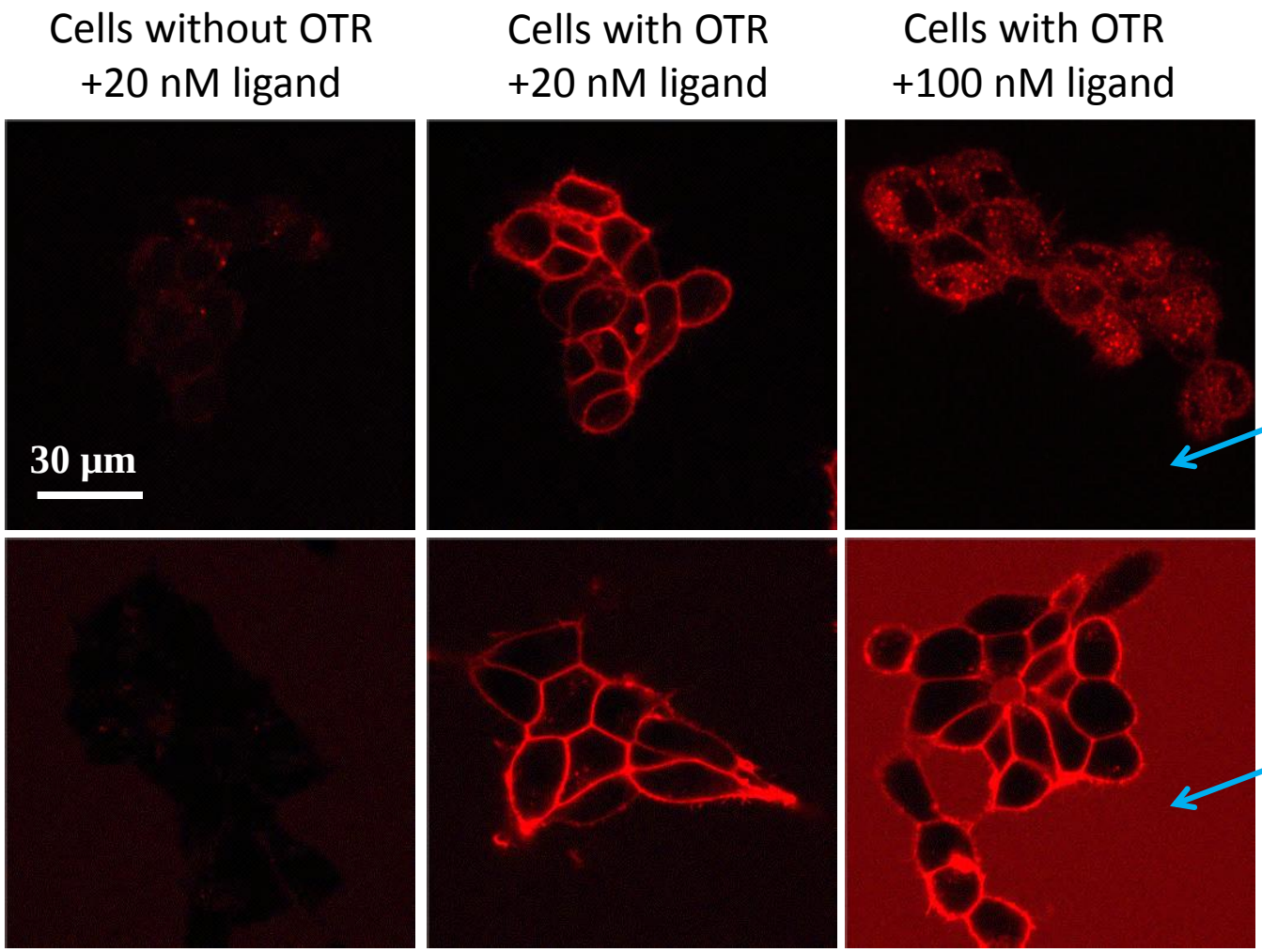


- ✓ New ligands turn on their fluorescence in the presence of receptor
- ✓ No effect is observed with classical ligand

Direct quantification: 29000 ± 5000 available receptors per cell
Independent radio-ligand method: 46000 ± 8000 receptors per cell

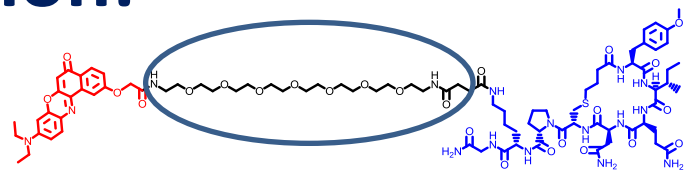
Karpenko et al., *ChemBioChem*, 2014, 15, 359.

Turn-on vs classical ligand: Fluorescence microscopy

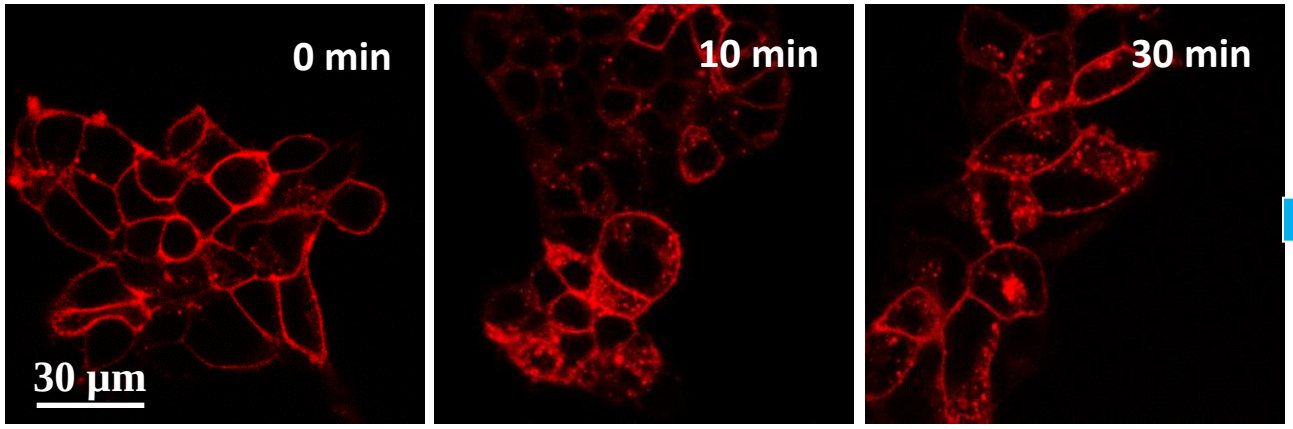


Turn-on ligand ensures receptor detection without background

Turn-on ligand internalization: Effect of spacer

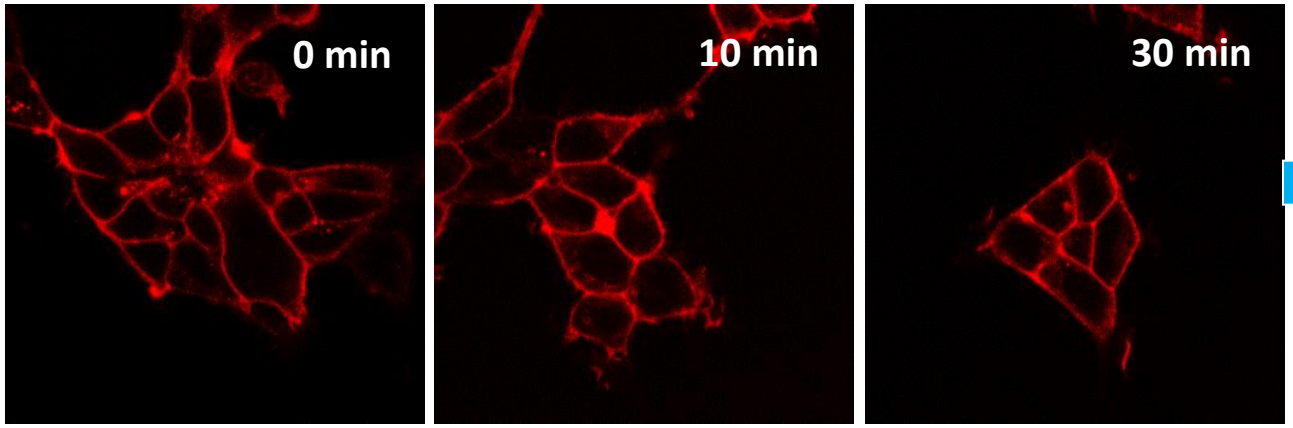


With Spacer



Agonist (with spacer)

Without Spacer



Antagonist (without spacer)

Long spacer preserves the agonist activity of carbetocin

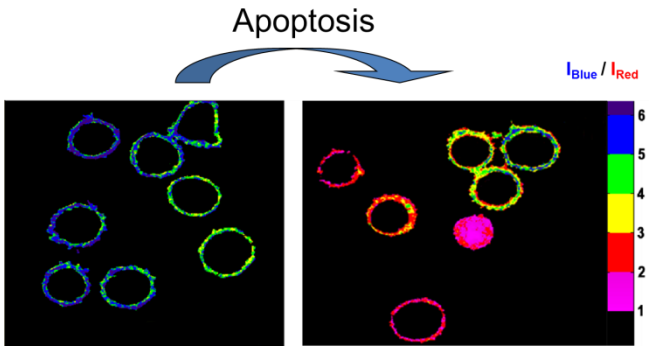
Karpenko et al., *ChemBioChem*, 2014, 15, 359.

CONCLUSIONS AND OUTLOOK

A tool kit for membrane research was developed based on environment-sensitive dyes:

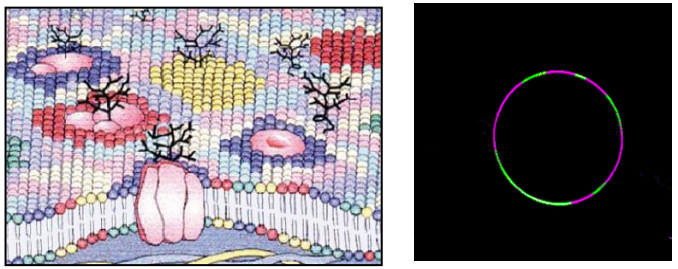
Probes for apoptosis

- ✓ Detection of transmembrane asymmetry
- ✓ Ratiometric response
- ✓ Suitable for screening pro-apoptotic agents
- ✓ In vivo applications?



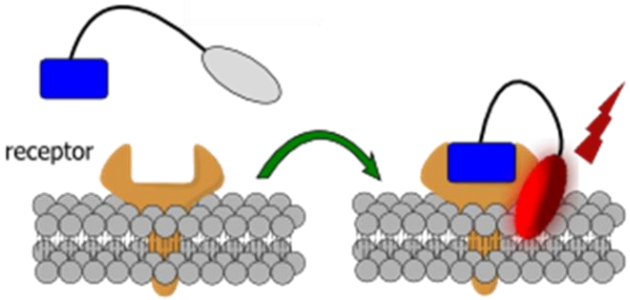
Probes for lipid order

- ✓ Imaging domains in model membranes
- ✓ Detection of cholesterol
- ✓ A phase-sensitive probe allows direct observation of lipid rafts in cells



Probes for membrane receptors

- ✓ Background-free detection of receptors
- ✓ Prospective for *in vivo* detection of overexpressed receptors in cancer cells



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- Sule Oncul

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- Alain Wagner, Nicolas Anton, Thierry Vandamme, UMR 7199 (Strasbrourg).
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