NEST

SCUOLA NORMALE SUPERIORE

Phasor-FLIM analysis of Doxorubicin liposomial formulations

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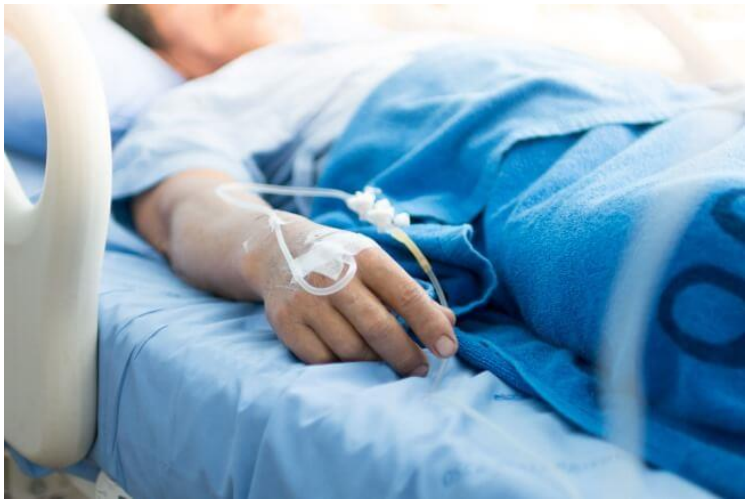
The encapsulated Doxorubicin: **Doxil®**

Generic Name: Doxorubicin liposomal

FDA approval: 1995

Drug Type: Doxorubicin is an anthracycline antibiotic used as chemotherapy drug.

Formulation: Three principal component: HSPC (phospholipid hydrogenated soy phosphatidylcholine), Cholesterol e DSPE (distearoyl-phosphatidylethanolamine) conjugated with PEG in a *ratio* 56:38:5

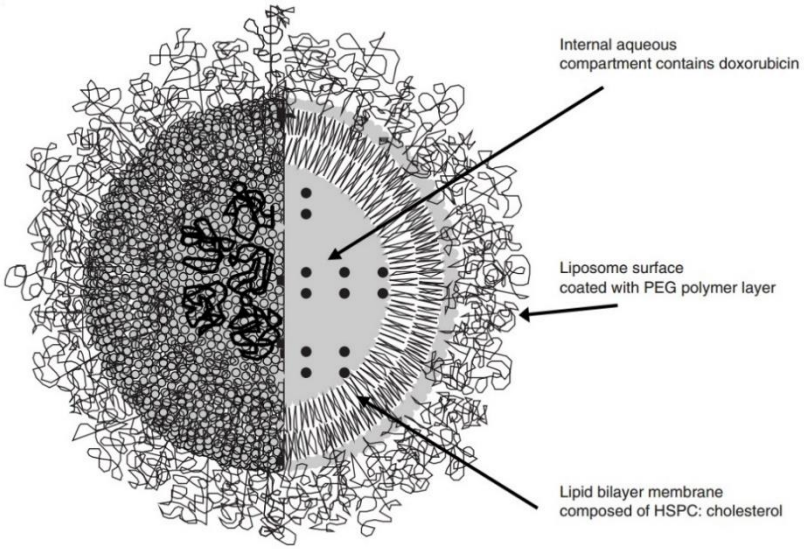


What Doxil Is Used For:
Doxil is used to treat AIDS-related Kaposi's sarcoma , breast cancer, ovarian cancer, and other solid tumors.

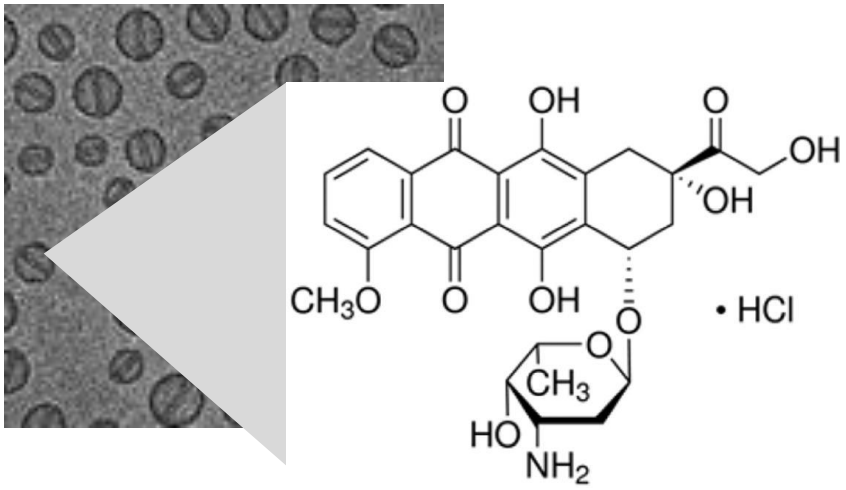
How Doxil Is Given:
- Doxil is given by injection through a vein (intravenously, by IV).
- There is no pill form of Doxil.
- The amount of Doxil needed for human treatment is ~50 mg/m^2

DOXIL: Doxorubicin liposomial formulation

In 1993 (patent) it was tought that Doxil was just free Doxorubicin in solution within the liposome...



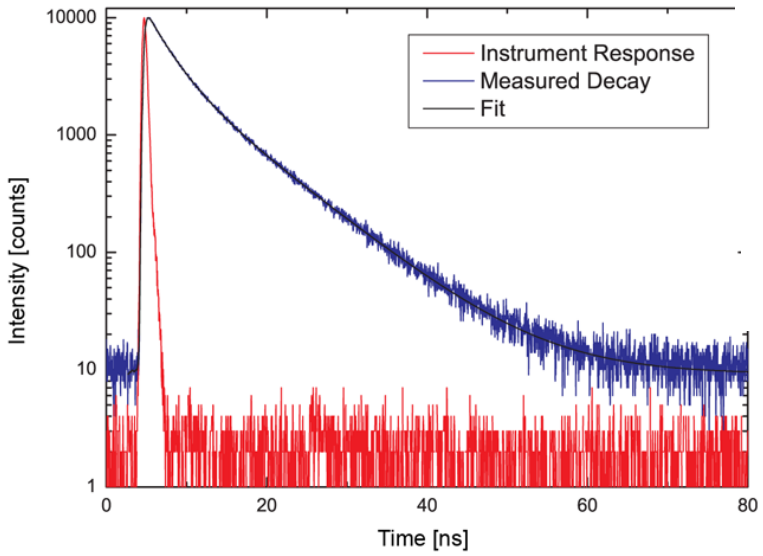
Today we know there is a crystal-like form in the liposome (together with free doxorubicin)



The formation of this structure is governed by the remote loading mechanism: this is based on continuous efflux of ammonia gas (formed by the pH-dependent dissociation of the intraliposomal NH_4^+ to neutral ammonia plus a proton), which creates a transmembrane pH gradient ($pH_{liposome} \ll pH_{medium}$). **The unionized doxorubicin that diffuses into the intraliposomal aqueous phase becomes protonated and can form intraliposome-insoluble doxorubicin-sulfate salt**. When the intraliposome doxorubicin concentrations are increased, the dox-sulfate-insoluble salt assembles into **nanorod crystals**

*Cardinal Role of Intraliposome Doxorubicin-Sulfate Nanorod Crystal in Doxil Properties and Performance, Xiaohui Wei et al., ACS Omega March 2, 2018

Fluorescence Lifetime Imaging Microscopy

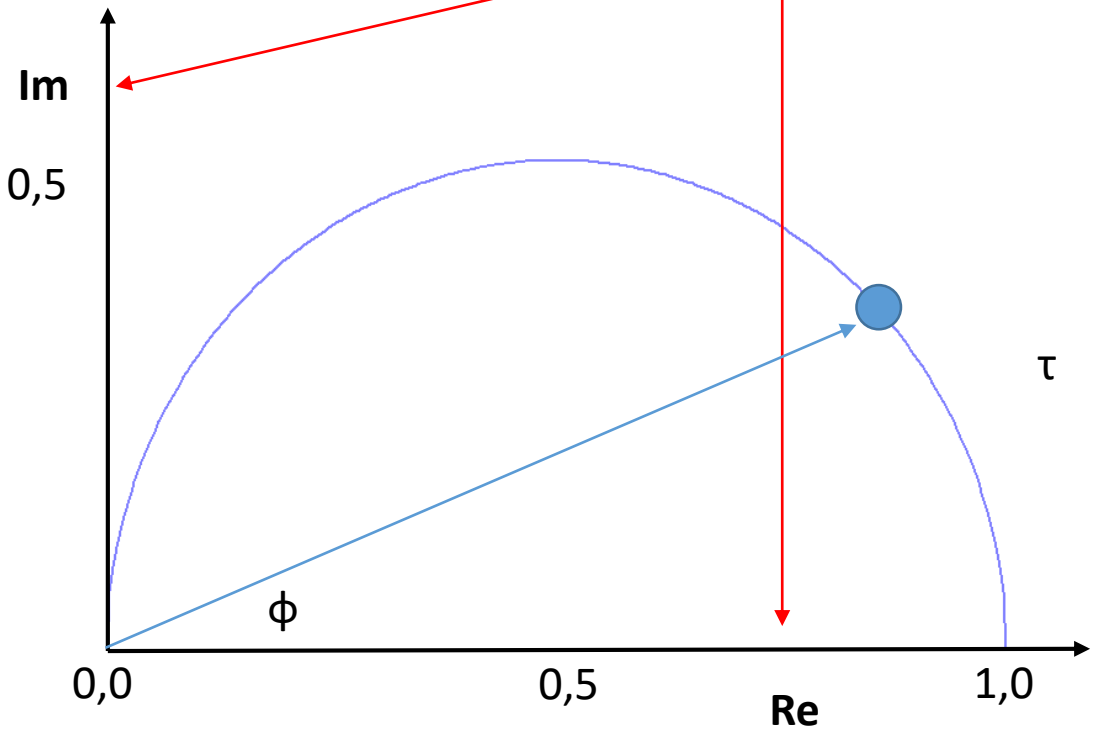


$$d(t) = \frac{1}{\tau} e^{-t/\tau}$$

Then the Fourier transformation at frequency ω is represented by the Lorentz function:

$$D(\omega) = \frac{1}{1 + j\omega\tau} = \frac{1}{1 + j\omega\tau}\frac{1 - j\omega\tau}{1 - j\omega\tau} = \frac{1 - j\omega\tau}{1 + (\omega\tau)^2} = \frac{1}{1 + (\omega\tau)^2} - j\frac{\omega\tau}{1 + (\omega\tau)^2}$$

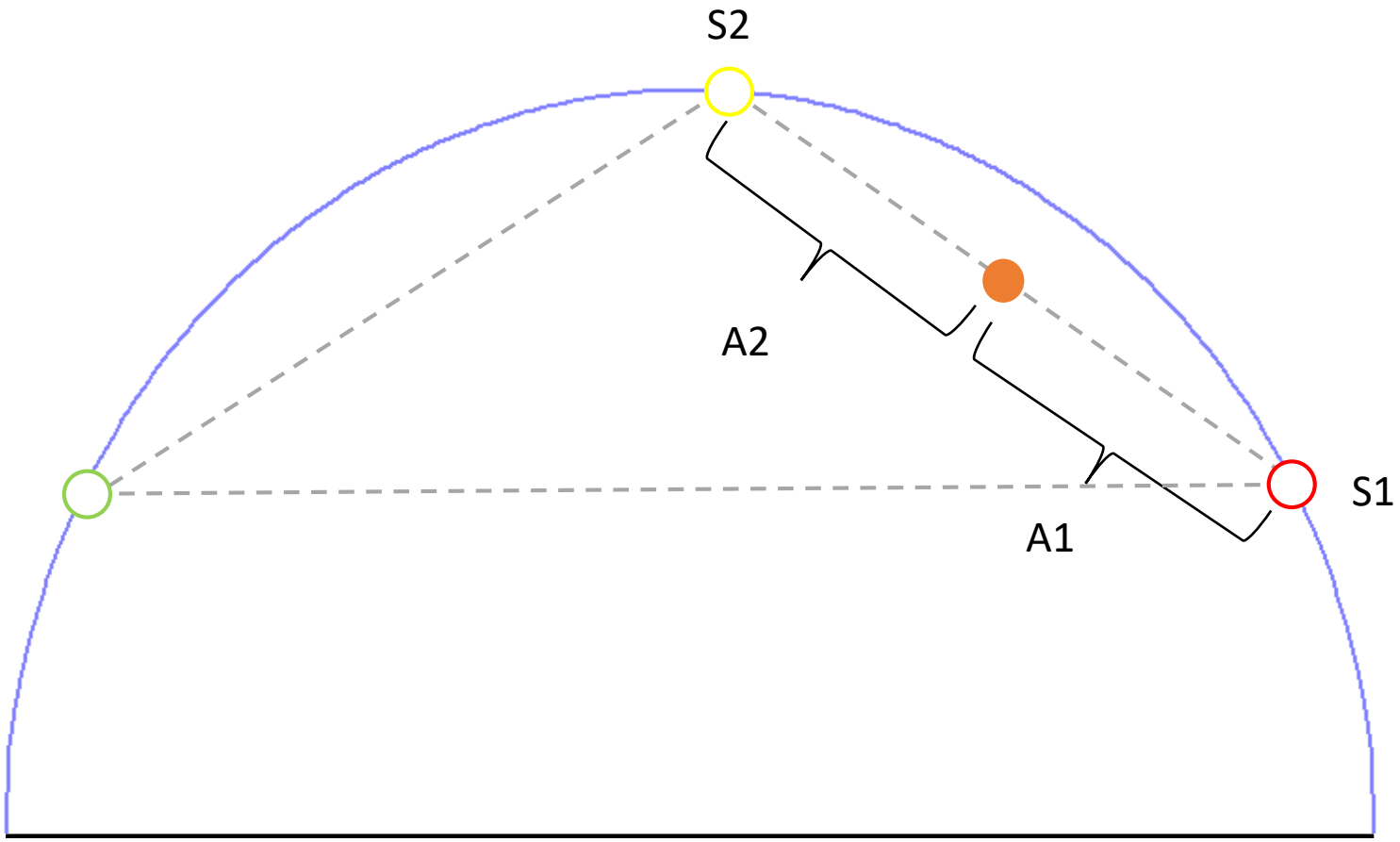
$$\tau = \frac{1}{\omega}\frac{\mathrm{Im}\, D(\omega)}{\mathrm{Re}\, D(\omega)}$$



The Phasor approach to lifetime analysis is fit-free, fast and provides a graphical representation of the measured curve

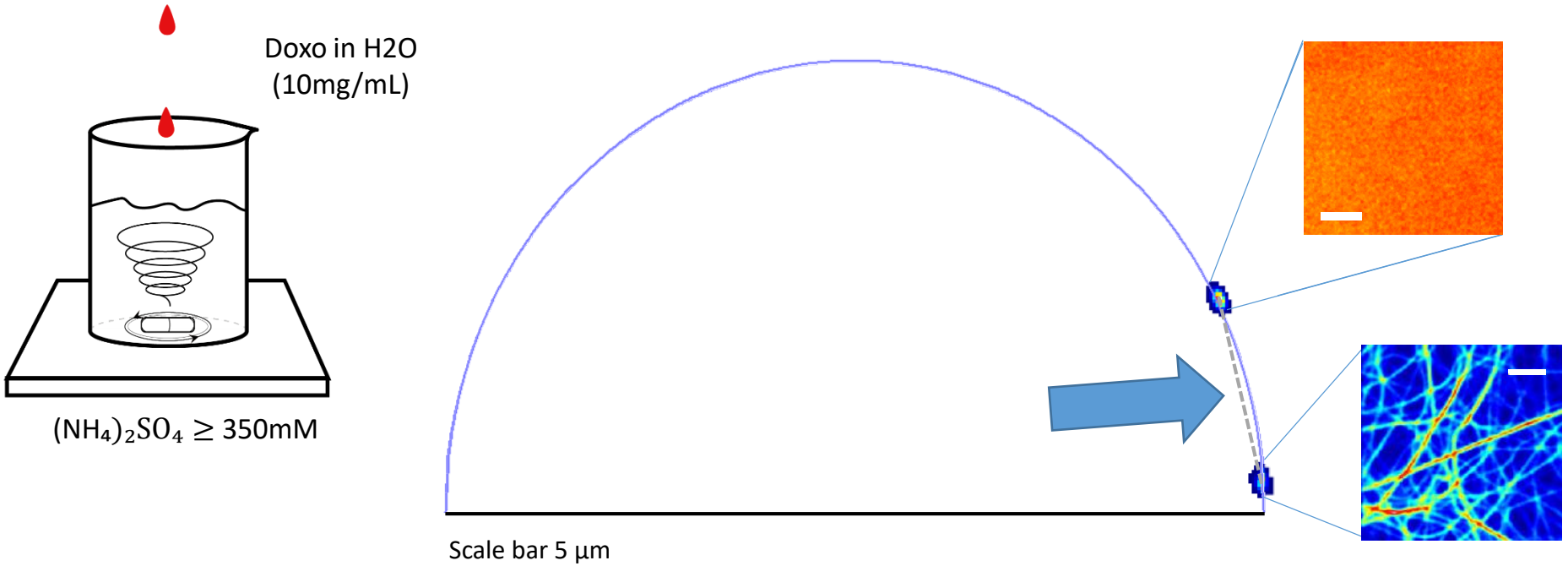
- **The phasor approach to fluorescence lifetime imaging analysis.** Digman MA, et al. Biophys J, 2008

Phasor Plot Rules



Fractional concentration of **Species 1**= (1 – A1)/(A1 + A2)

Doxorubicin Nanorods



First step: measure the life-time of the free form of Doxo by FLIM (Fluorescence-lifetime imaging microscopy)

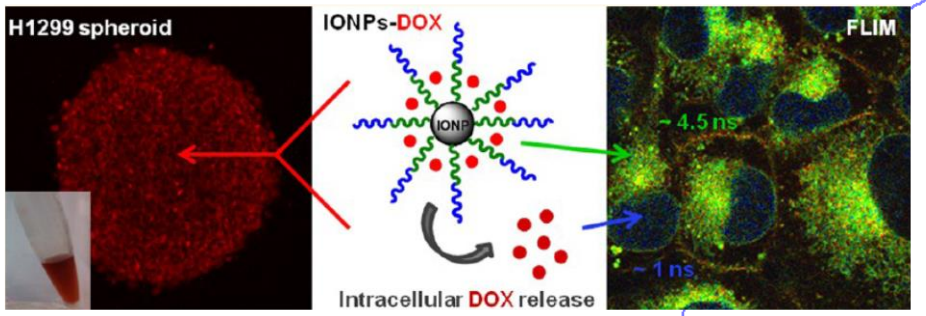
Second step: synthesize the nanorods structure of the doxorubicin.

- The protocol by Xiaohui Wei et al* was used: Doxorubicin HCl in water(10 mg/mL) was added dropwise to a solution of ammonium sulfate (500mM) under magnetic stirring.

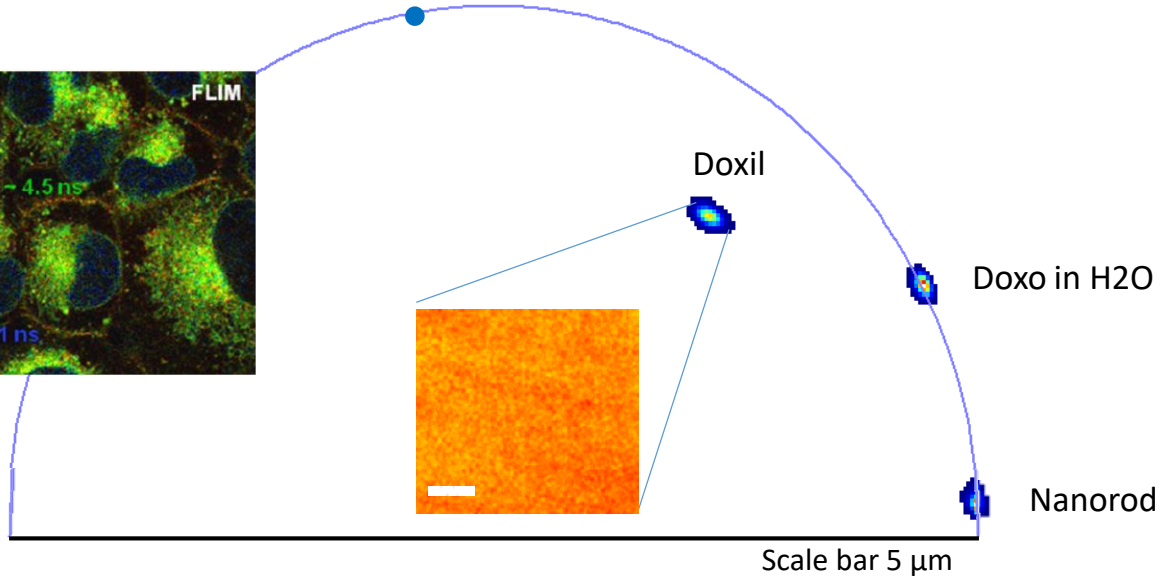
The resulting precipitation was measured by FILM

*Cardinal Role of Intraliposome Doxorubicin-Sulfate Nanorod Crystal in Doxil Properties and Performance, Xiaohui Wei et al., ACS Omega March 2, 2018

But the phasors of the Doxil are in a completely different position.



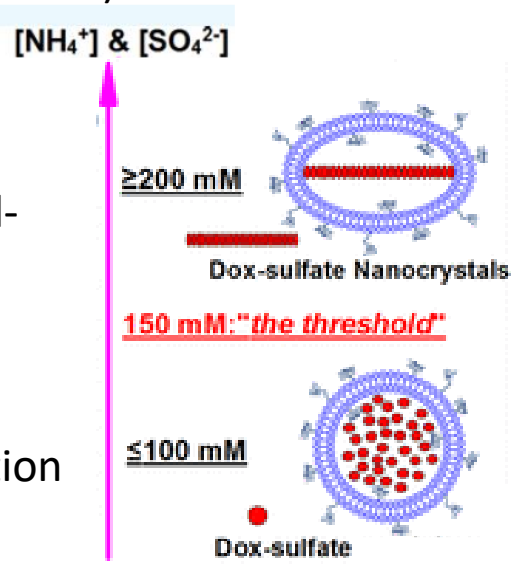
- Basuki et al ACS nano 2013



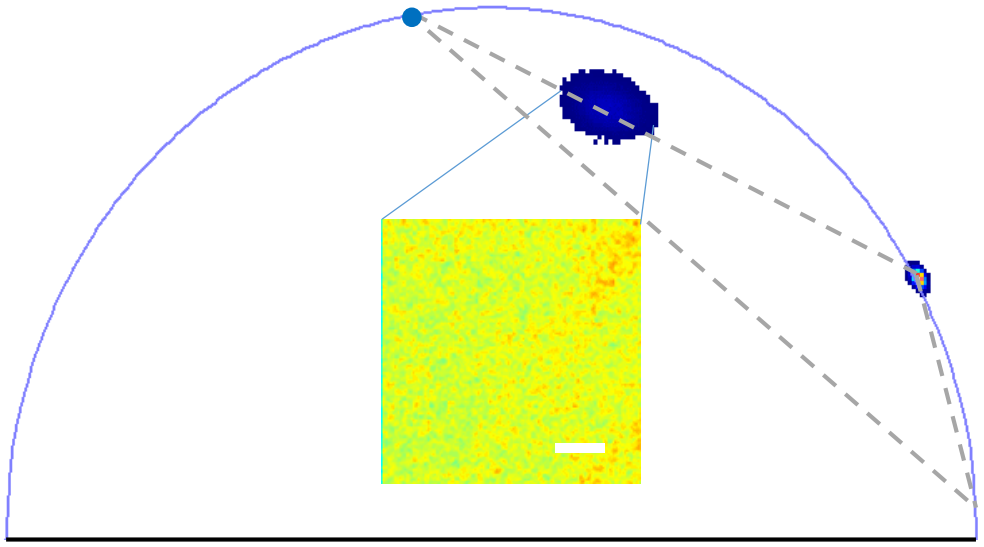
So we postulate the existence of a third component. From the literature, it is known that doxorubicin bound to a surface has lifetime at about 4.6 ns (blue dot)

We theorized that this point is the consequence of the conjugation of the free Doxo with the lipid bilayer since they are the only molecules available. To prove that the synthesis of doxil-like particles without the nanorods structures is required. To achieve that the protocol described by Xiaohui Wei et al* was used

So the loading was executed with low intraliposomial concentration of $(NH_4)_2SO_4$ (≤ 100 mM)



The lifetime of «simil» **Doxil®**:

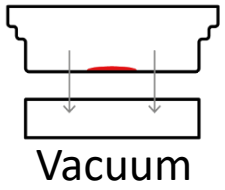


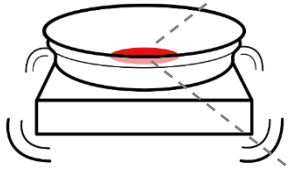
Scale bar 5 µm

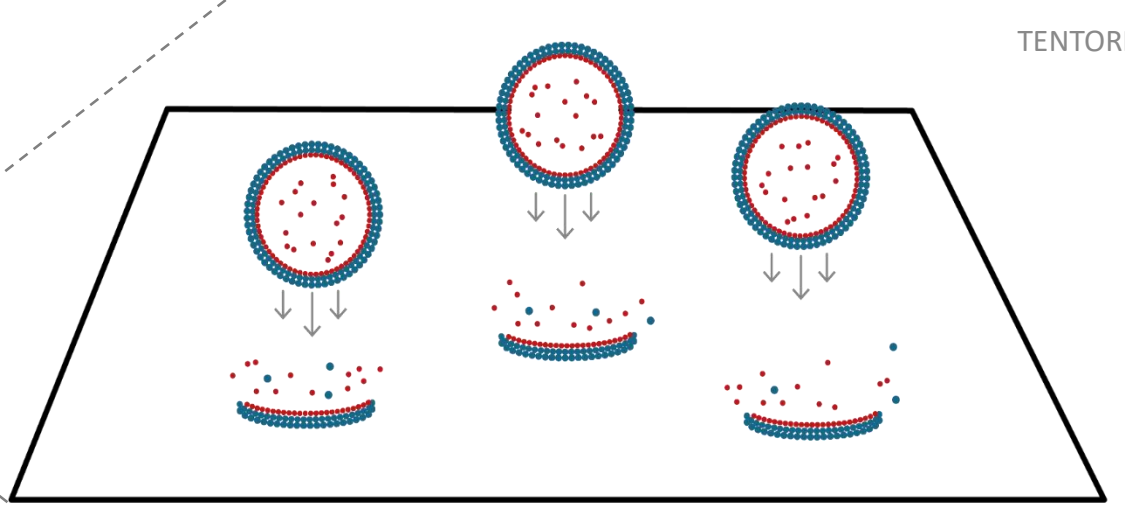
It is possible to see that the phasors of this doxil-like particles are on the line of conjunction between the free Doxo and the theoretical point.

For the phasor plot rules seems that our interpretation could be the correct, but further experiment are required

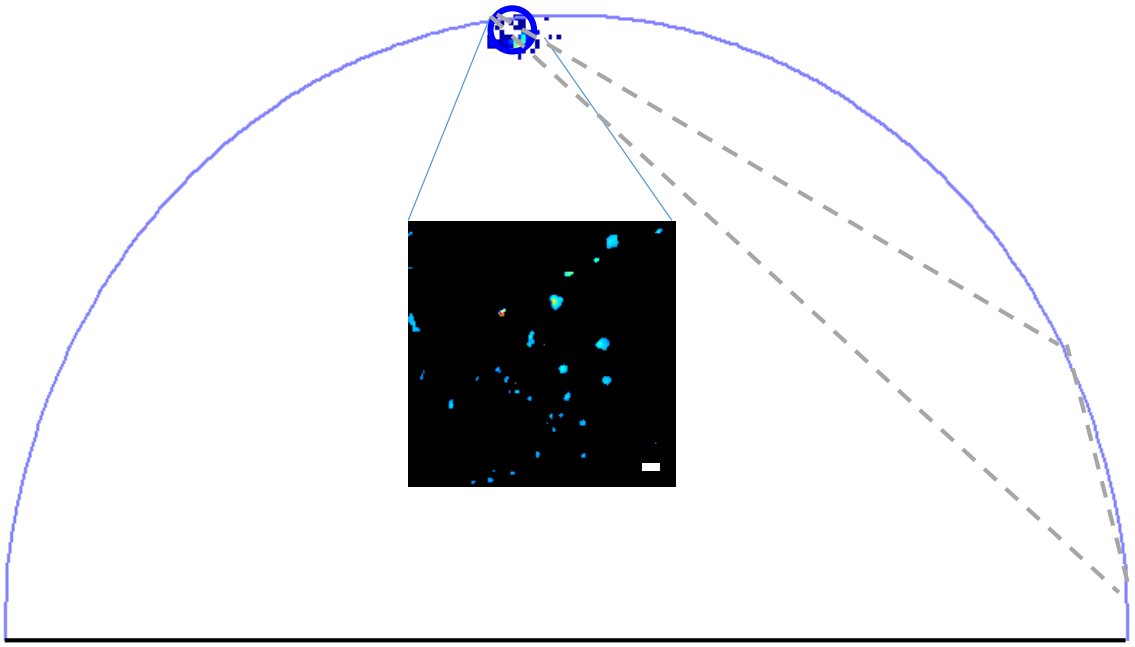
Spin Coater







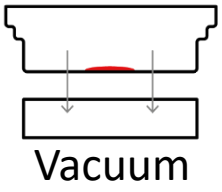
The spin coater is used to mechanically destroy the liposome and isolate membrane patches with the associated Doxorubicin molecules. The glass is removed and analyzed by FLIM

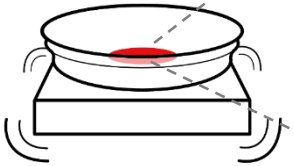


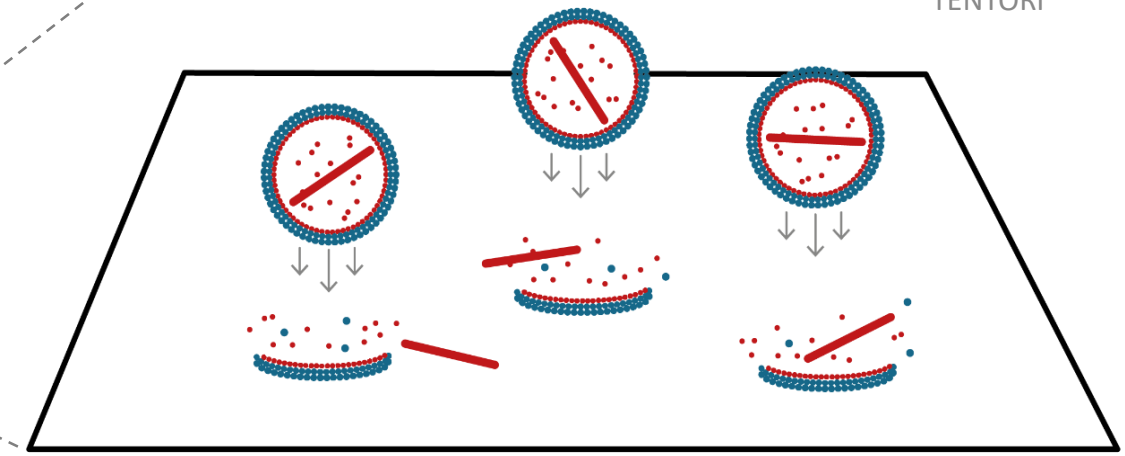
Scale bar 10 µm

The theoretical point is now experimentally confirmed

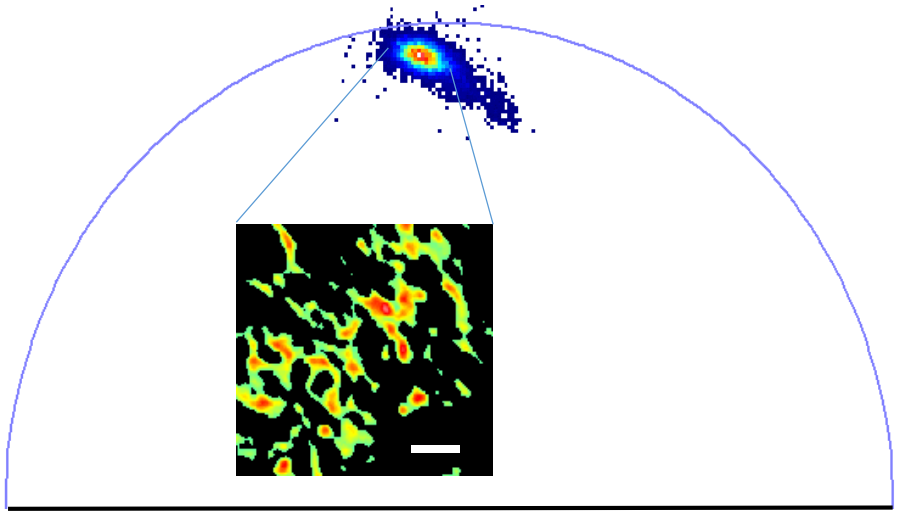
Spin Coater

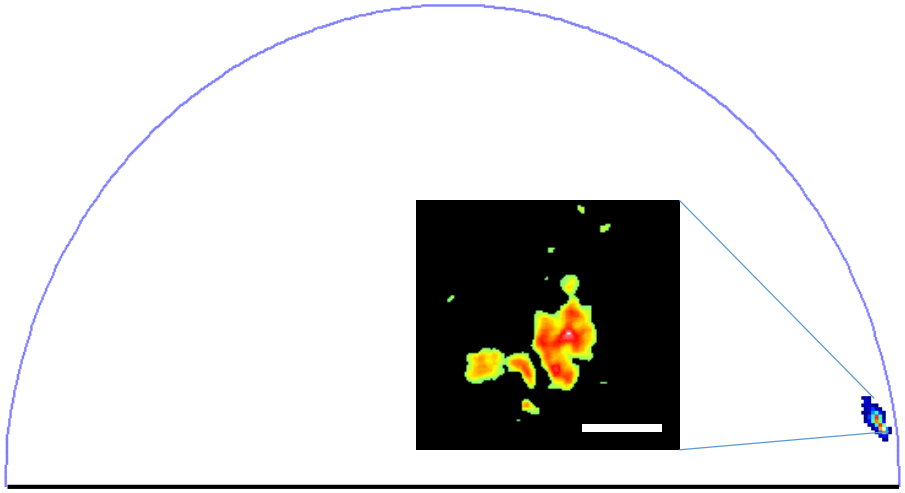






The spin coater was used again with the Doxil for further confirmation

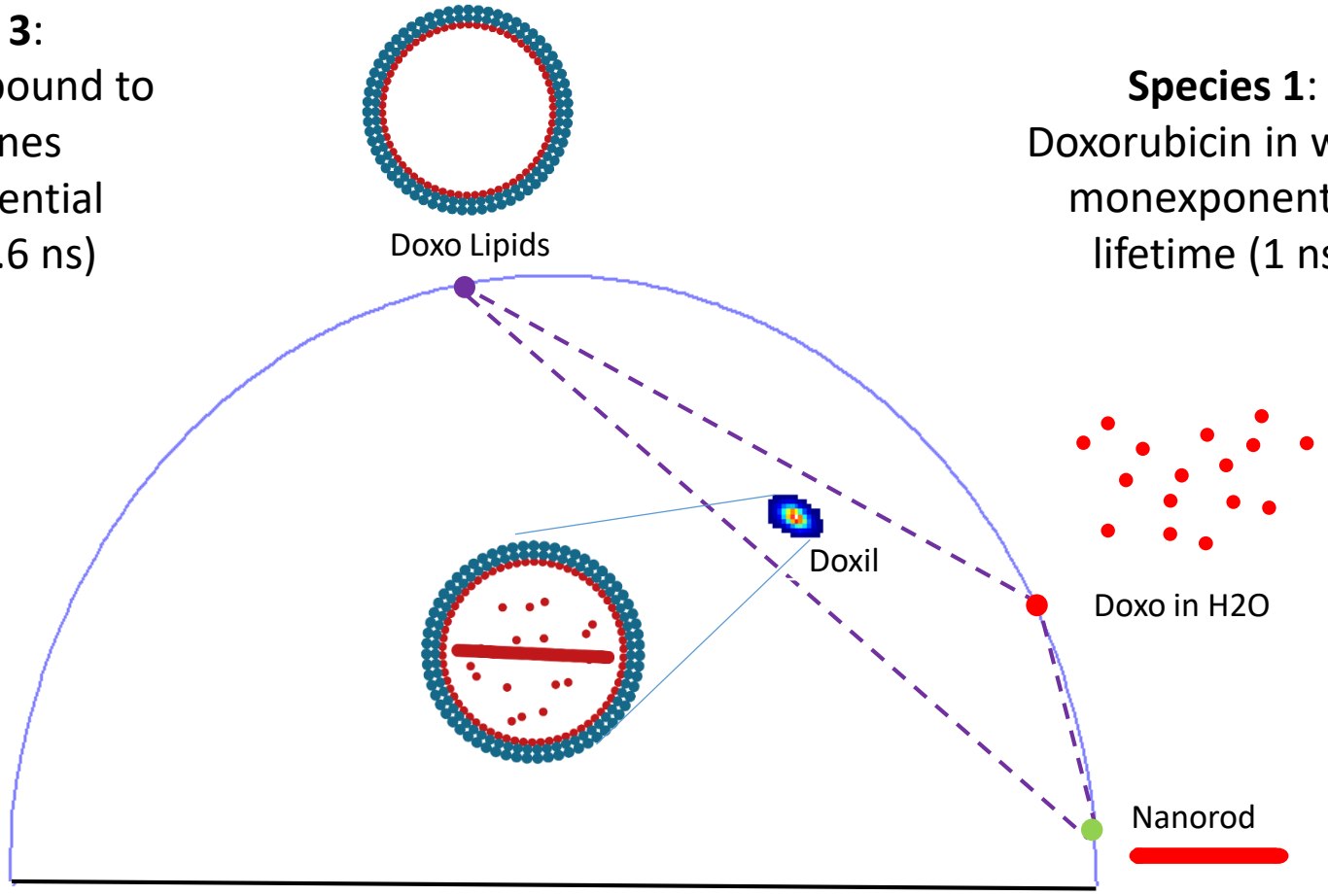




Scale bar 2 µm

Possible model:

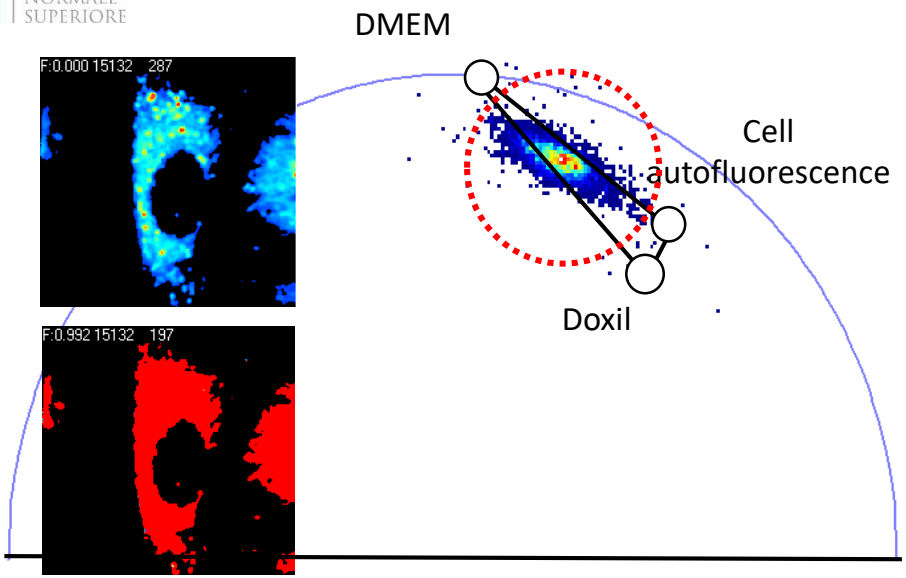
Species 3:
Doxorubicin bound to membranes
monexponential
lifetime (4.6 ns)



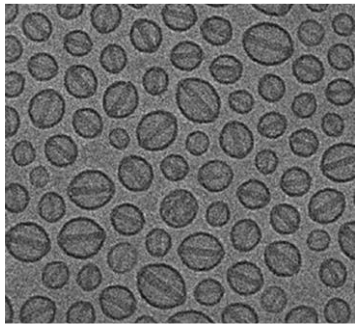
Species 1:
Doxorubicin in water
monexponential
lifetime (1 ns)

Species 2:
Doxorubicin nanorods
monexponential
lifetime (0.3 ns)

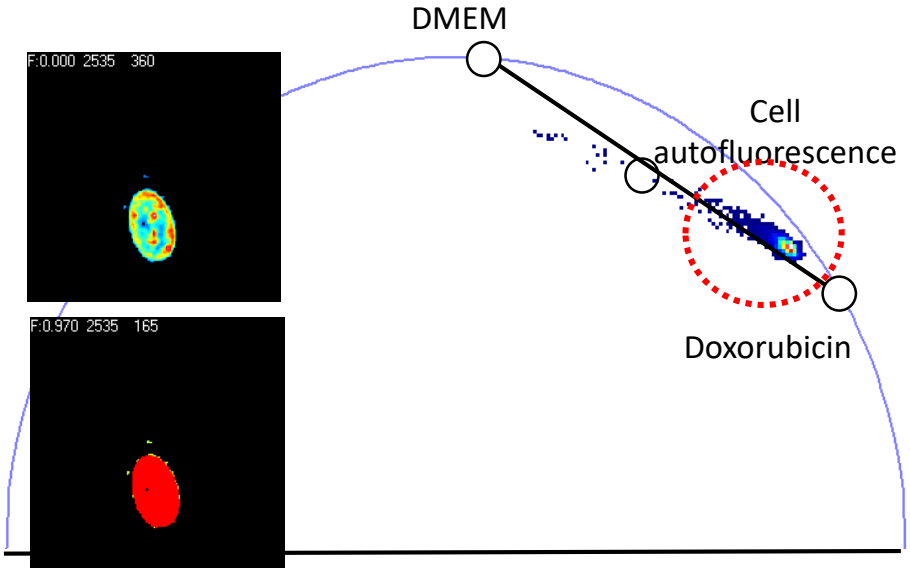
Doxil® vs Doxorubicin in cells:

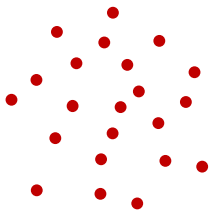


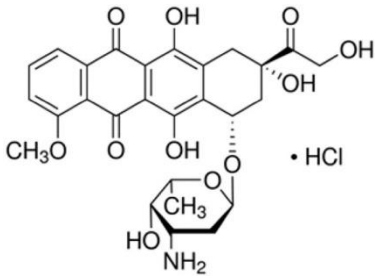
No corona



Transient exposure
(4h, then whashing)

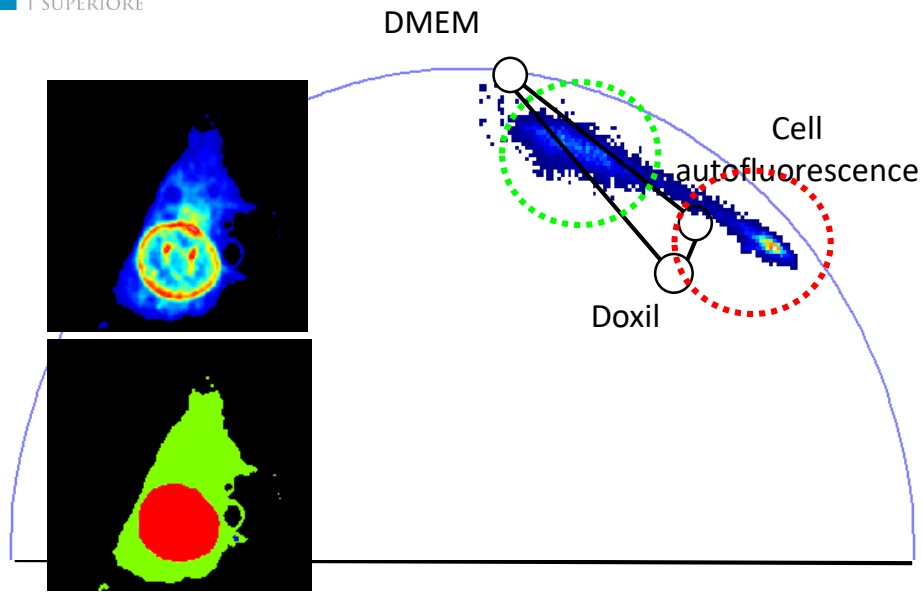




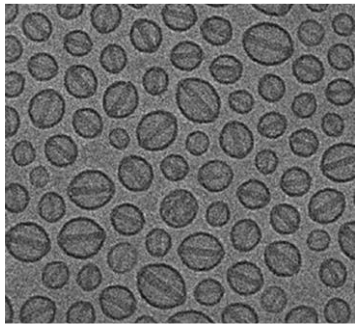


Free Doxorubicin

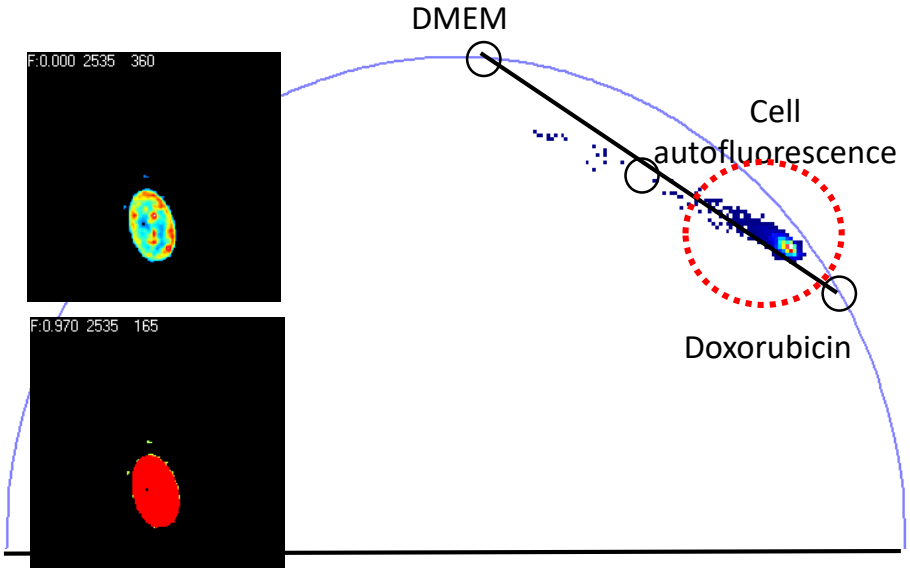
Doxil® vs Doxorubicin in cells:

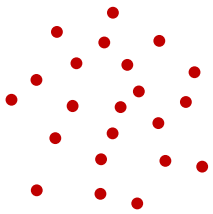


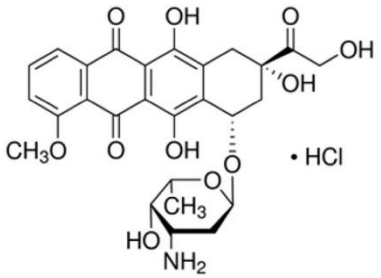
No corona



Permanent exposure
(no whashing)







Free Doxorubicin