Confocal Microscopy

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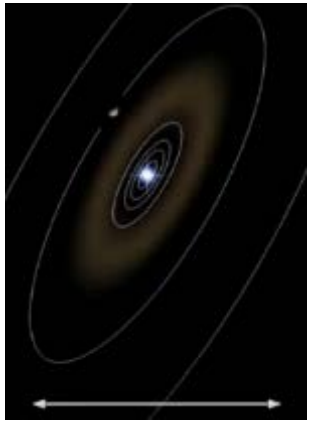
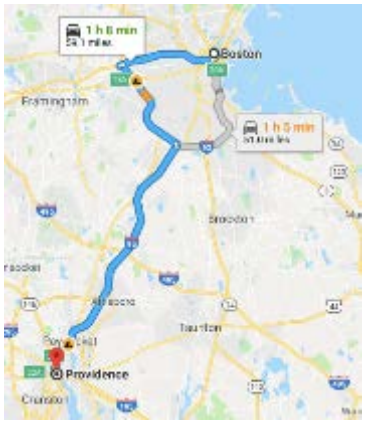
The Size of Things

The Solar System



1 billion meters

New England

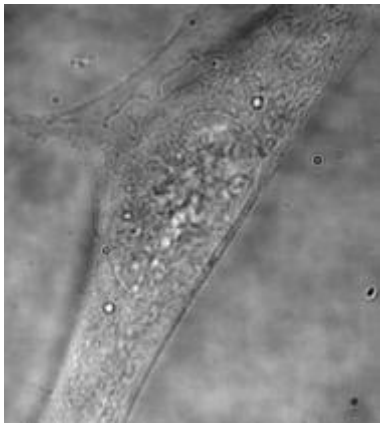


100 thousand meters

Humans

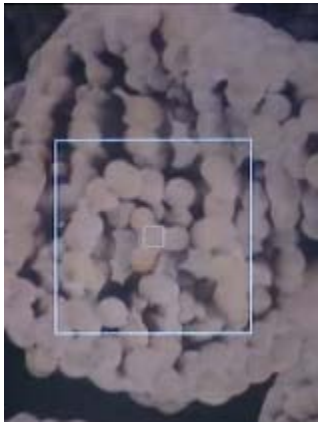
1 meter

Cells



1 / 100 thousand meters

Molecules



1 / billion meters

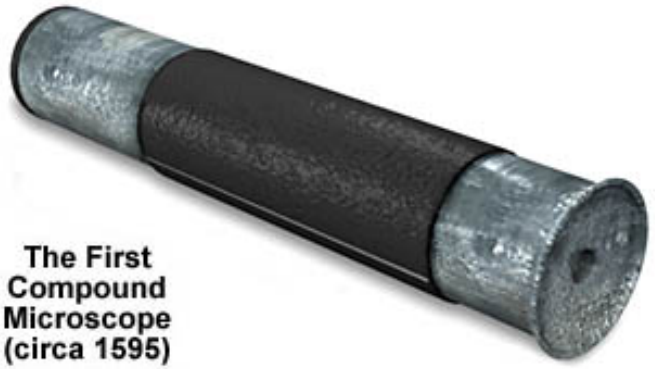
History of Microscopes

History of Microscopes

Zacharias Janssen
(1580-1638)

- The first microscope was discovered around 1595 by Hans and Zacharias Janssen.

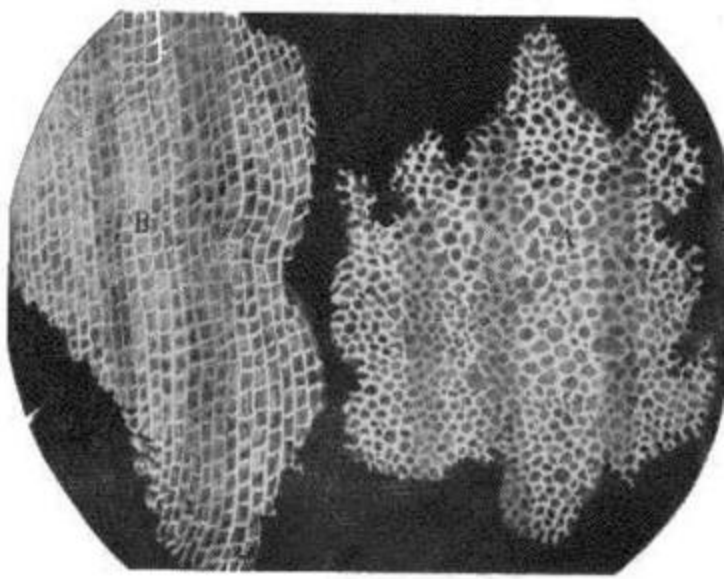
- This microscope consisted of simply lenses adapted to the ends of the tube. It could enlarge objects 3 times-9 times.



The First
Compound
Microscope
(circa 1595)

History of Microscopes

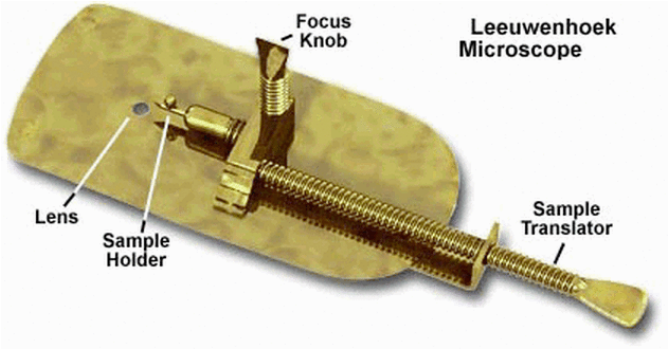
•In 1665, Robert Hooke first used the term 'cell'. He called the holes in the bottle cork he looked at as 'cells'.



•According to the rumors, when he looked carefully at the cell mushroom, he remembered the rooms where the priests stayed and used this name

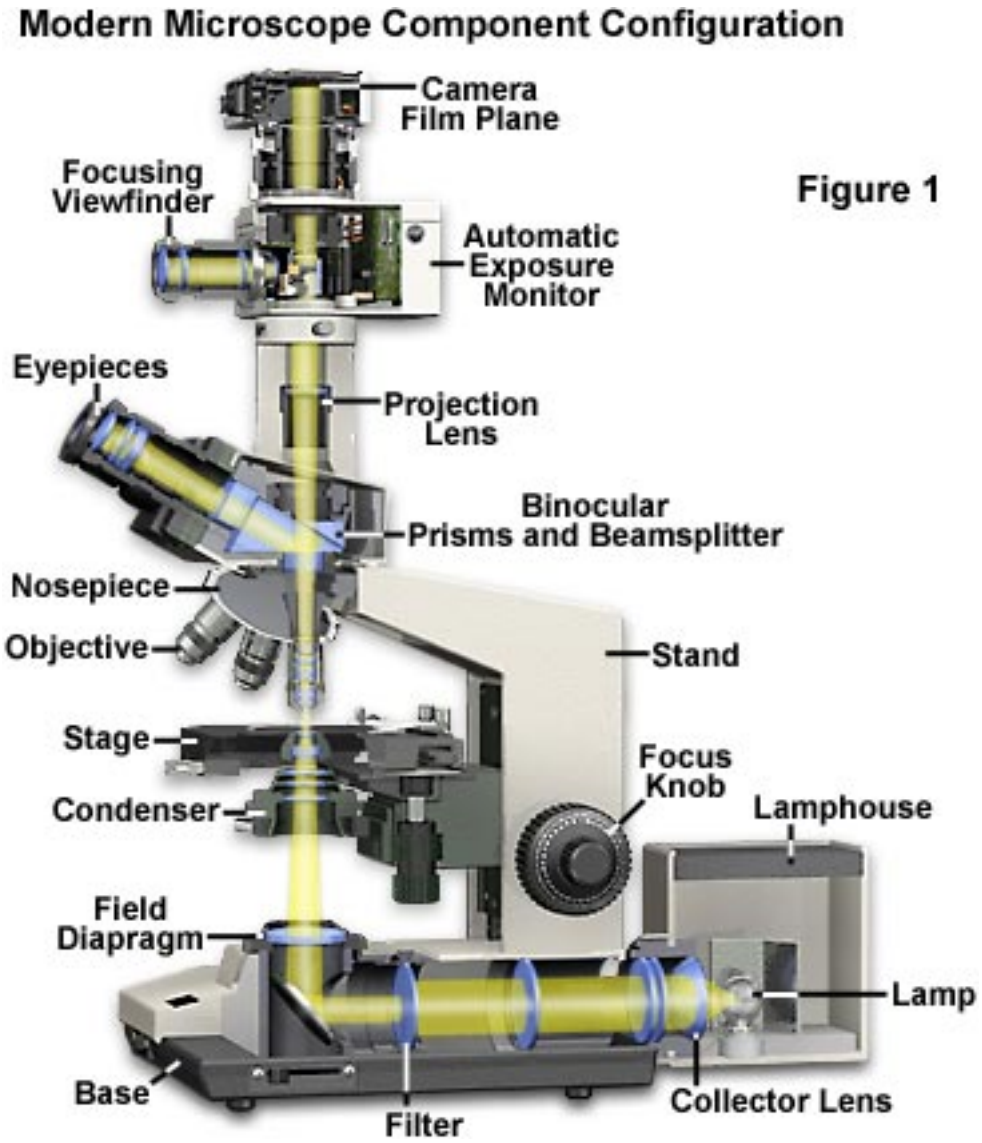
History of Microscopes

- Immediately after Hooke (circa 1683), Anton Van Leeuwenhoek, a Dutch amateur scientist, observed the first living cells under a microscope.



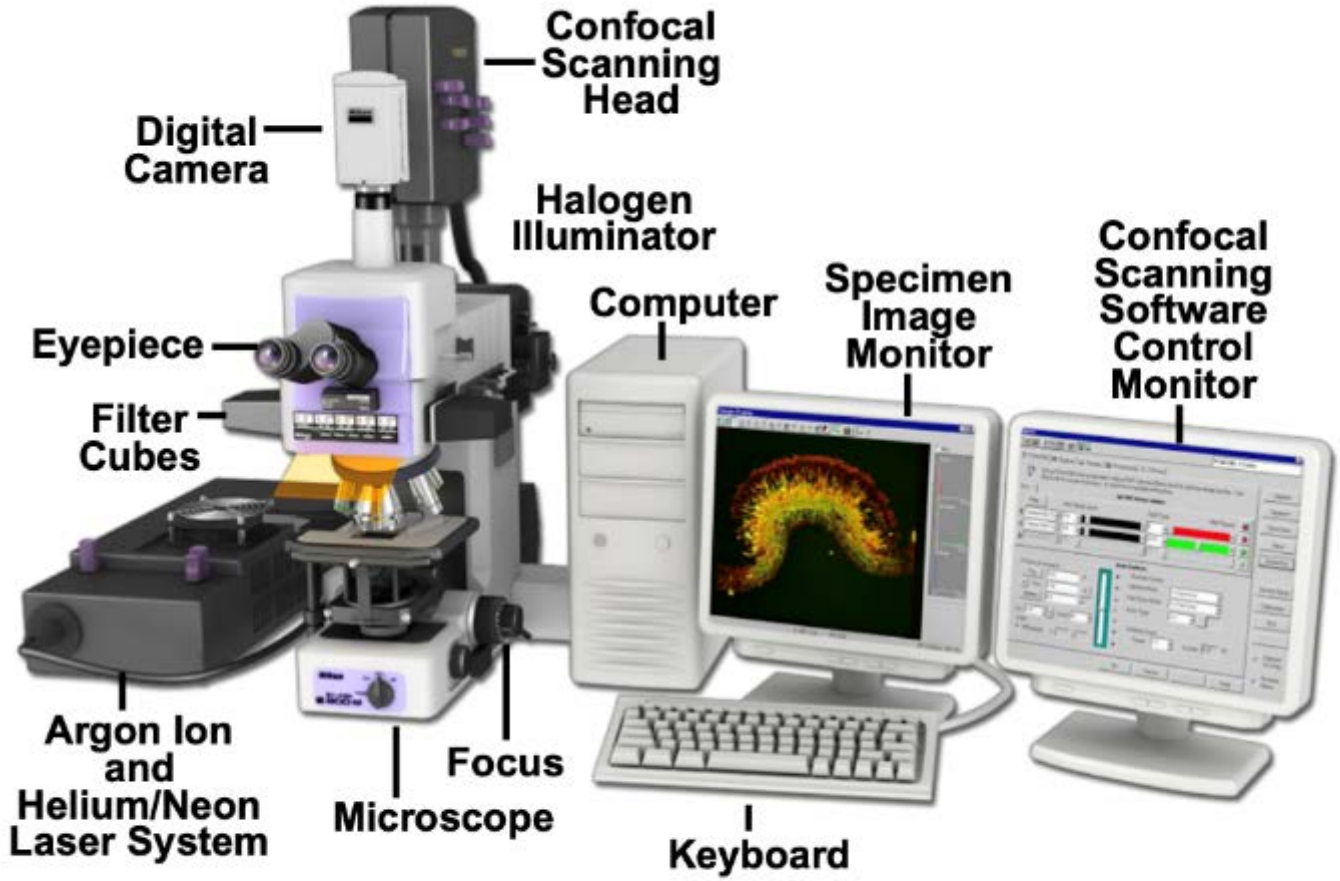
Modern Microscope

Modern Microscopy



https://www.microscopyu.com/techniques/confocal/introductory-confocal-concepts

Laser Scanning Confocal Microscopy



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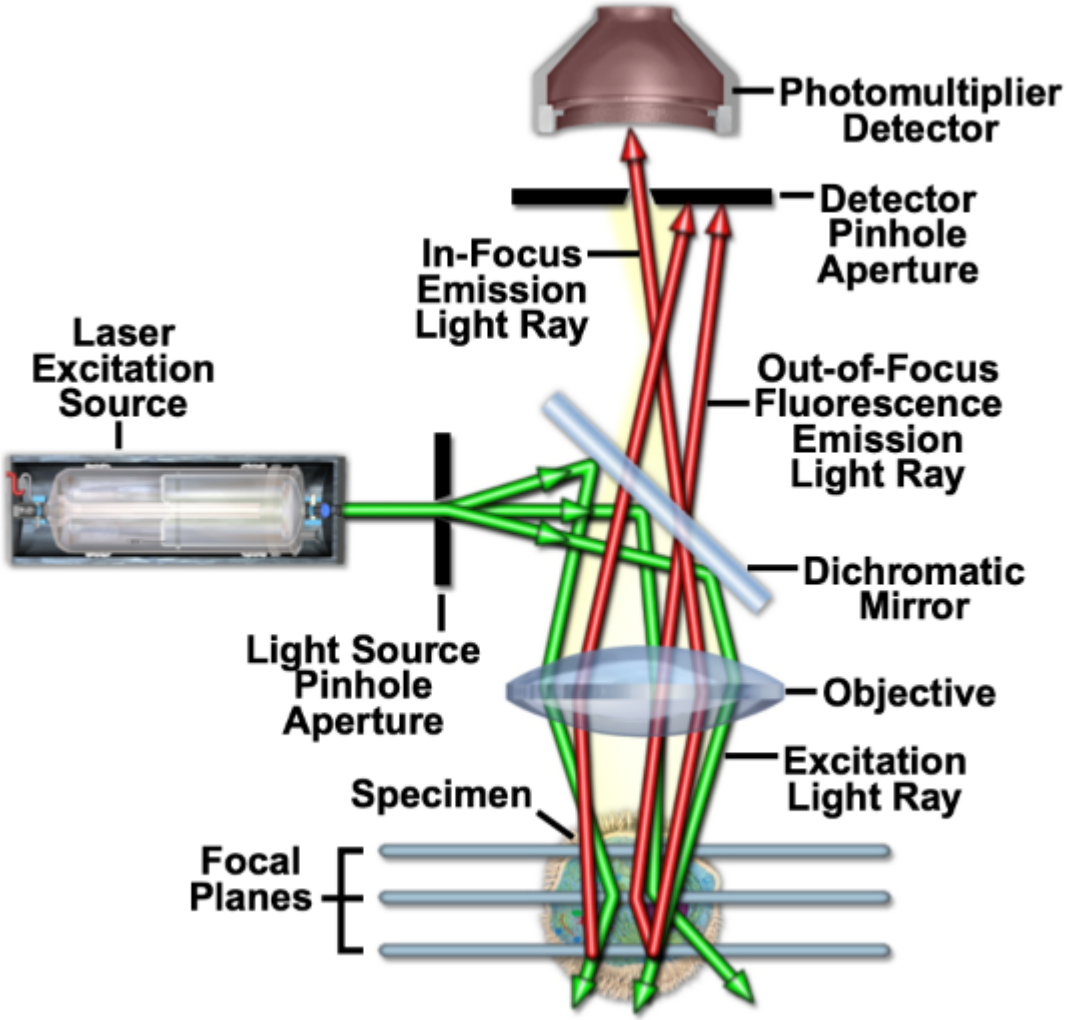
Laser Scanning Confocal Microscopy



Principal Light Pathways in Confocal Microscopy

https://www.microscopyu.com/techniques/confocal/introductory-confocal-concepts

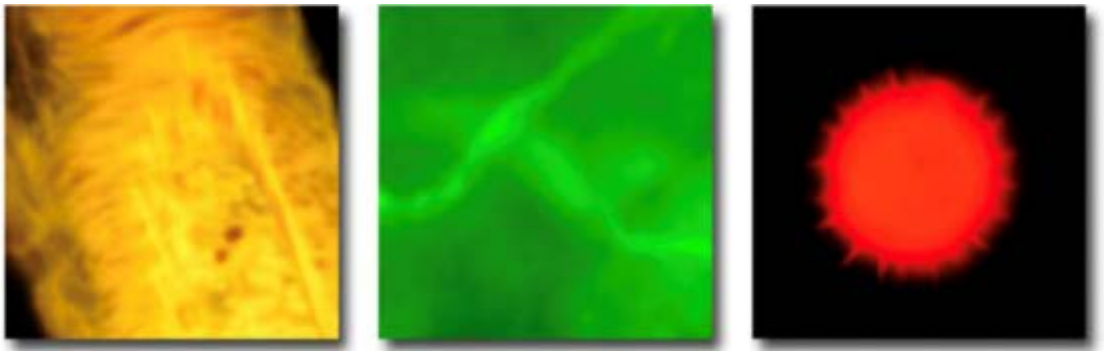
Laser Scanning Confocal Microscopy

Widefield versus Point Scanning of Specimens

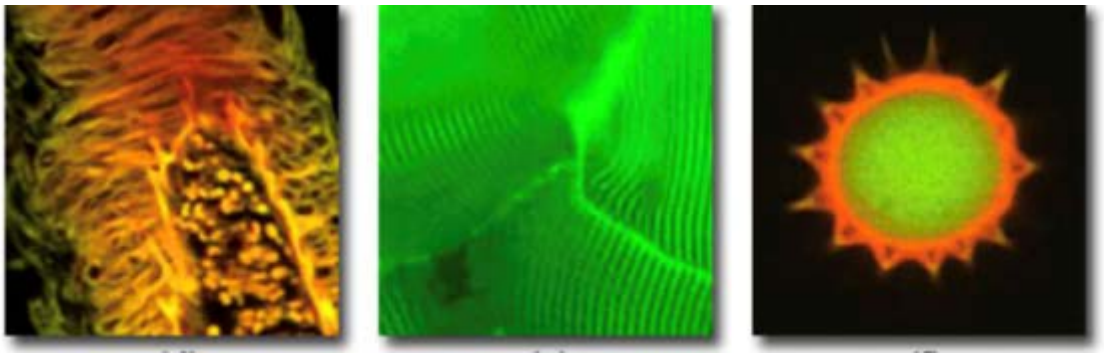
https://www.microscopyu.com/techniques/confocal/introductory-confocal-concepts

Laser Scanning Confocal Microscopy

Widefield Microscopy



Confocal Microscopy



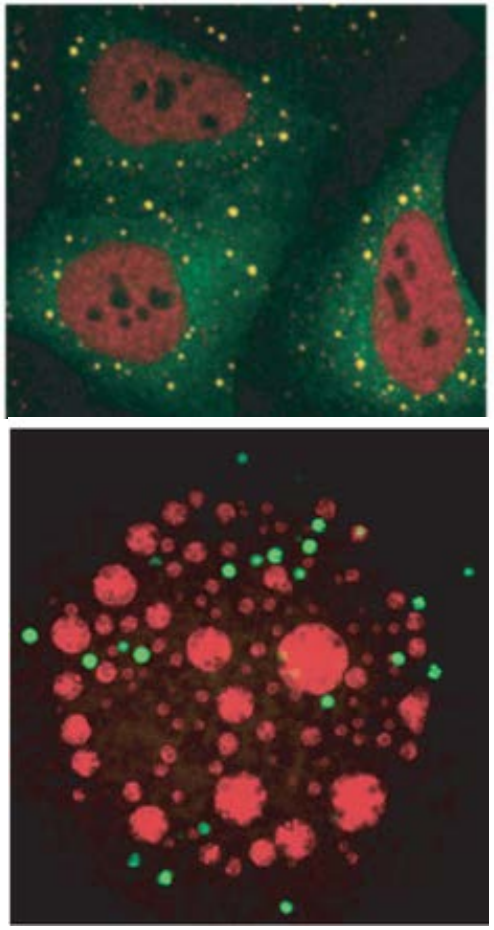
https://www.microscopyu.com/techniques/confocal/introductory-confocal-concepts

Diffusion of Biomolecules in Vesicle Coated RNA-Based Coacervates

Activities in cell can be compartmentalized by phase separation

- Aqueous phase separation observed in vivo
  - Cytoplasmic and nucleoplasmic
  - Membraneless organelles
  - Composed of RNA and protein

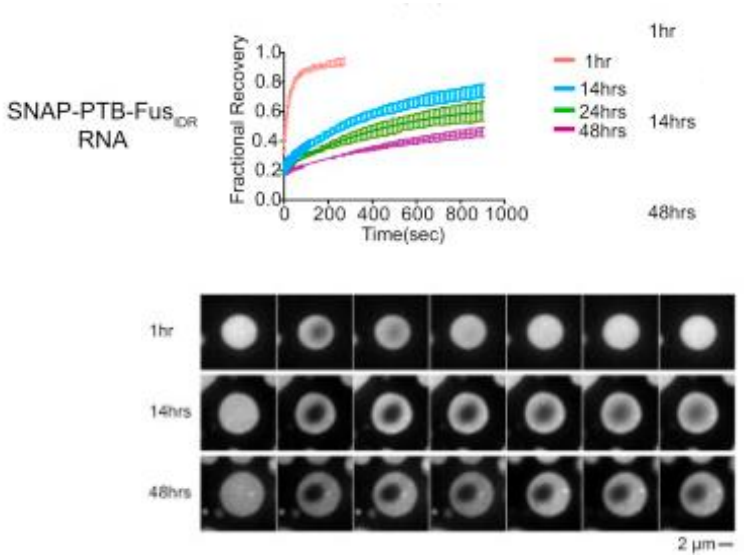
- Consequences
  - Biomolecule partitioning
  - Colocalization
  - Regulation of metabolism



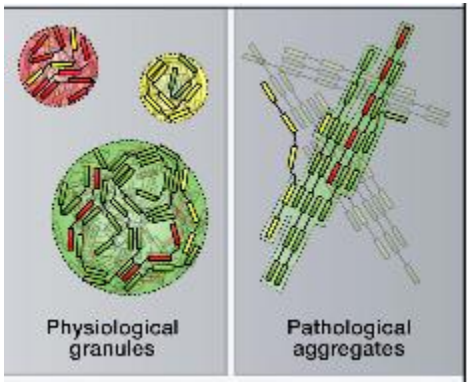
Brangwynne et al. Nat Physics. 2015;11:899.

Diffusion of biomolecules indicates dynamic structure

- Rapid recovery of fluorescence is used to define liquid like droplets in vivo.

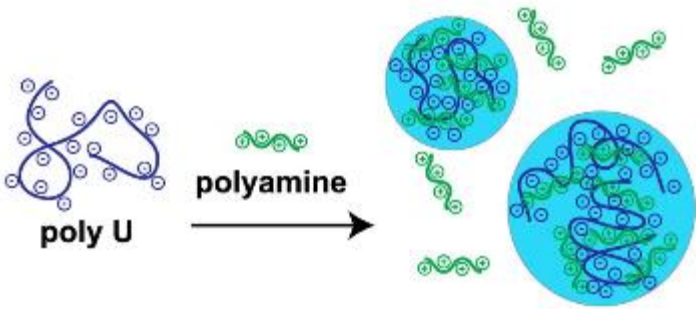


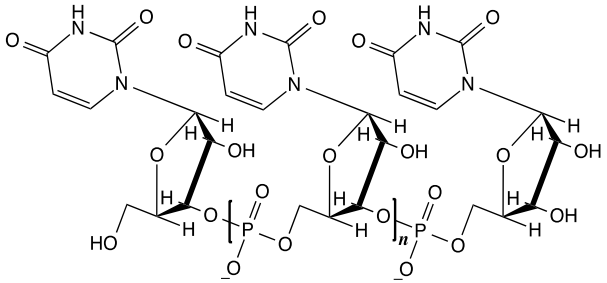
- Liquid-like properties of droplets could facilitate function



Lin, Yuan, et al. *Molecular cell*, 2015, 208-219.
Weber, Stephanie C. et al. *Cell* 2012, 1188-1191.

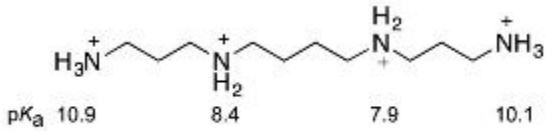
PolyU/spermine coacervate system



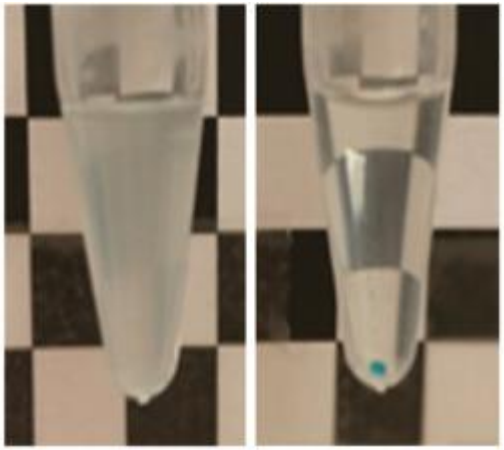


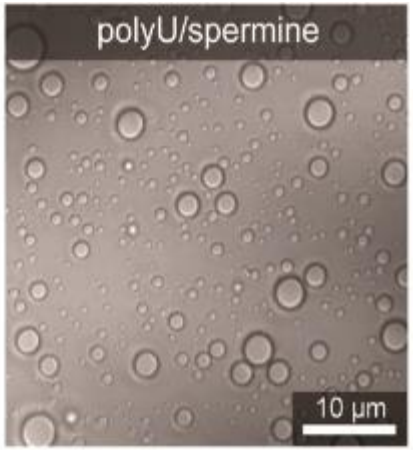
pk_a ≈1

Poly(uridylic acid)
MW=~ 600-1000 kDa
N~ 2600



Spermine

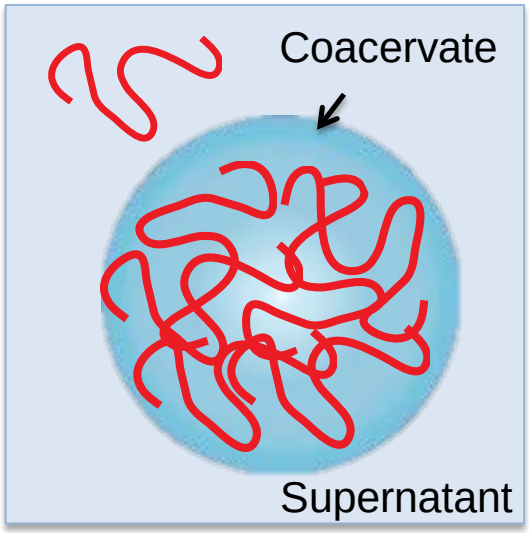




- Low complexity RNA (polyU) and polyamines (spermine) formed coacervates.
- Centrifugation of samples resulted in a very small coalesced coacervate phase at the bottom.

Aumiller Jr, W. M., **Pir Cakmak, F.,** Davis, B. W., & Keating, C. D., *Langmuir*, *32*(39), 10042-10053.

Partitioning in coacervate



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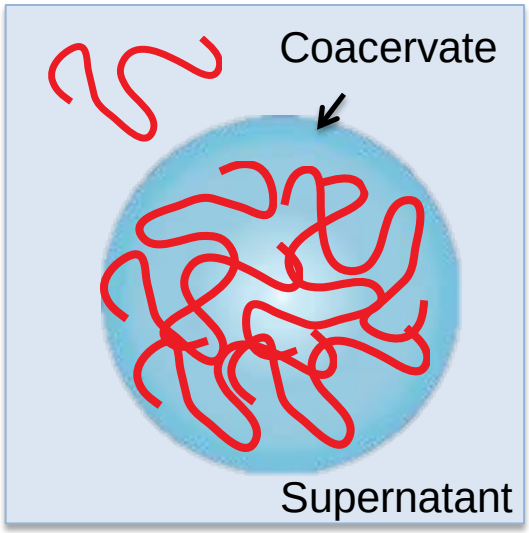
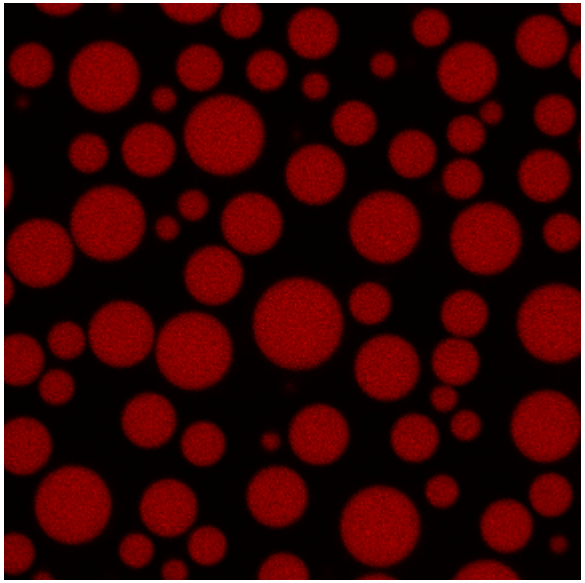
1855

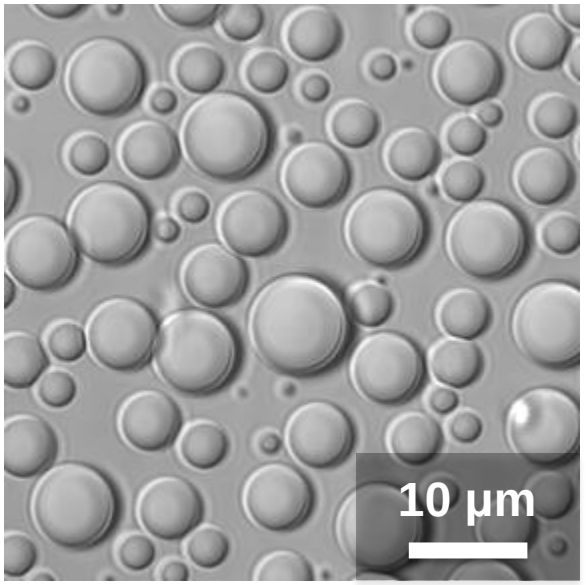
Concentration in the droplet can be calculated using microscopy



Fluorescence



Brightfield



Biomolecule is labelled with fluorescence dye

Fluorescence intensity in the droplets are compared to calibration curves to obtain concentration value.

Calibration curves are made with know concentration values of labelled molecule at the same conditions.

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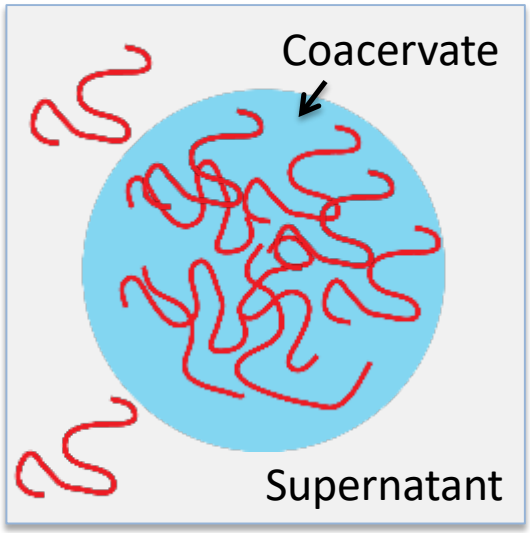
Partitioning in PolyU/spermine coacervate system



$C_{coacervate}$= Concentration in coacervate droplet

$C_{Supernatant}$= Concentration in supernatant phase

$$K = \frac{C_{Coacervate}}{C_{Supernatant}}$$

Poly U15- 15 nucleotides composed of Uridine- *Has the weakest binding with PolyU*

Poly N15- 15 nucleotides composed of random nucleotides

Poly A15- 15 nucleotides composed of Adenosine-*Has strongest binding due to base pairing*

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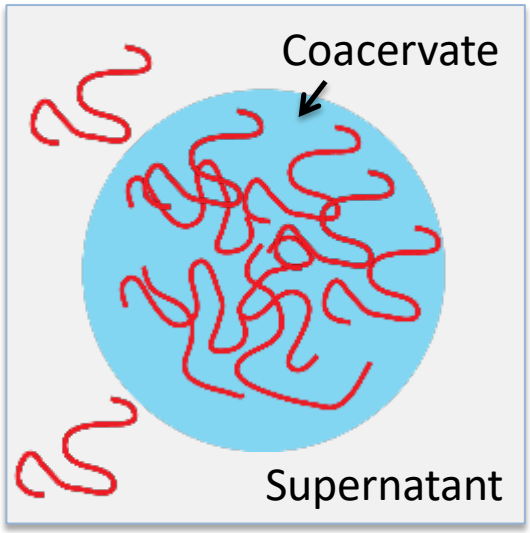
Partitioning in PolyU/spermine coacervate system



$C_{coacervate}$= Concentration in coacervate droplet

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	Poly A15	Poly N15	Poly U15
Partitioning coefficient (K)	2800	62	44

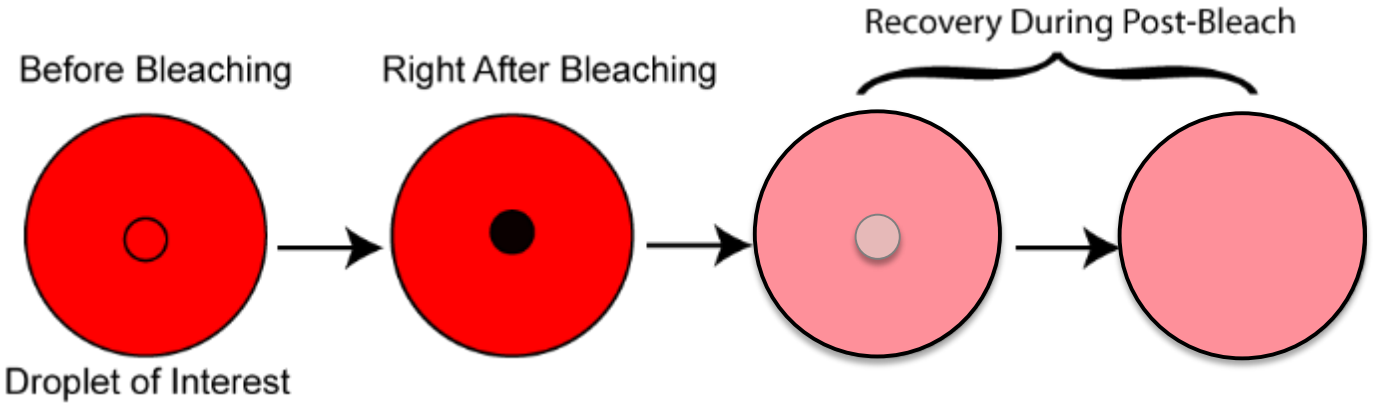
Poly U15- 15 nucleotide composed of Uridine- *Has the weakest binding with PolyU*

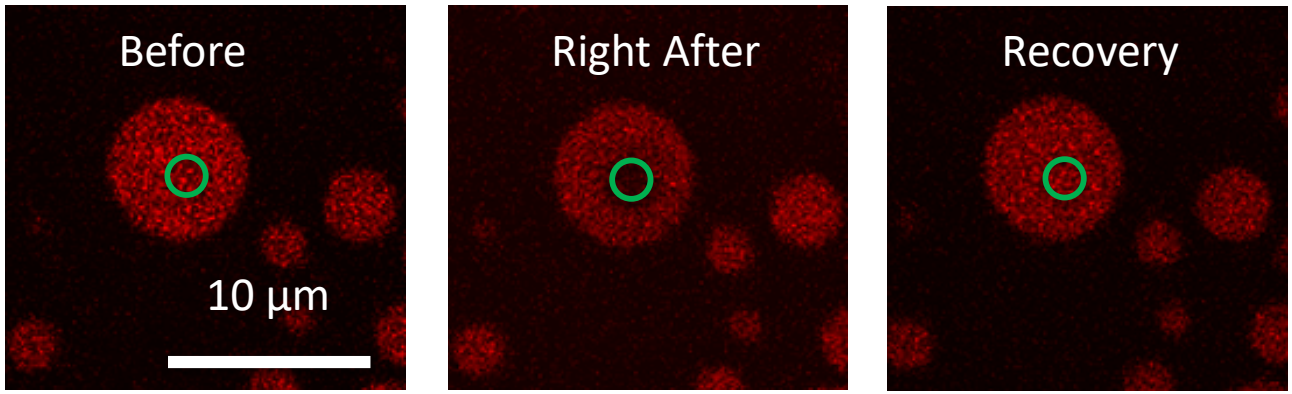
Poly N15- 15 nucleotide composed of random nucleotides

Poly A15- 15 nucleotide composed of Adenosine- *Has strongest binding due to base pairing*

Partial Diffusion - What is Fluorescence Recovery After Photobleach (FRAP)?

Partial Droplet Bleaching





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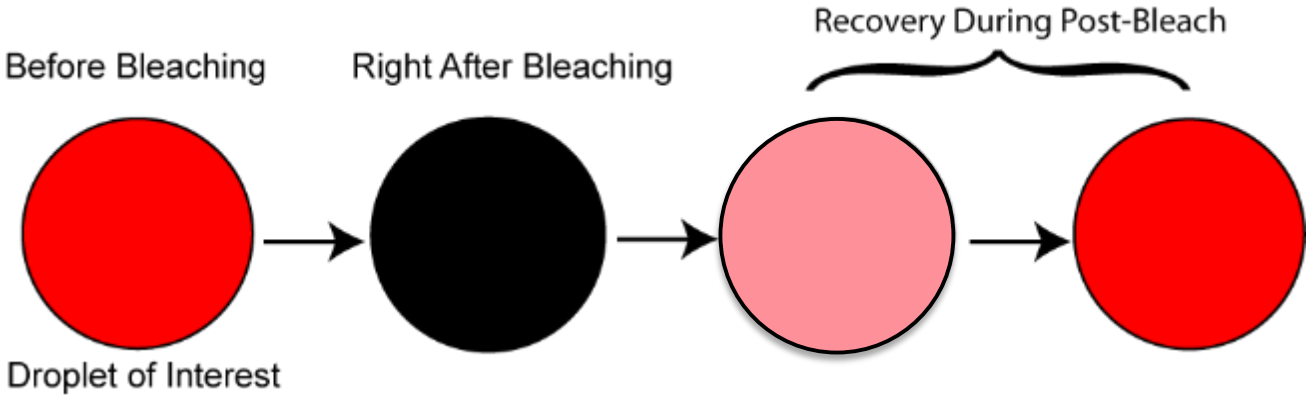
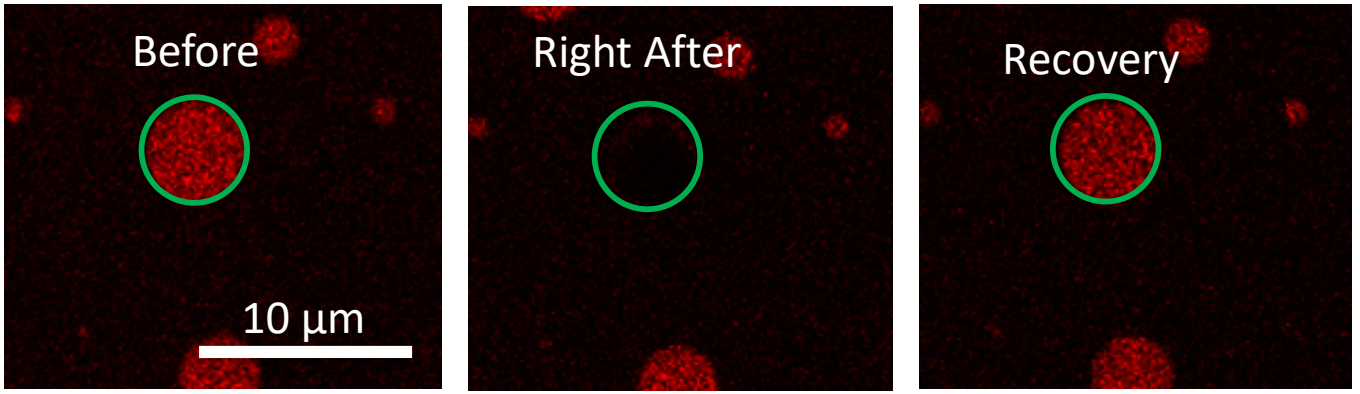
Entire Droplet Diffusion- FRAP

Entire Droplet Bleaching





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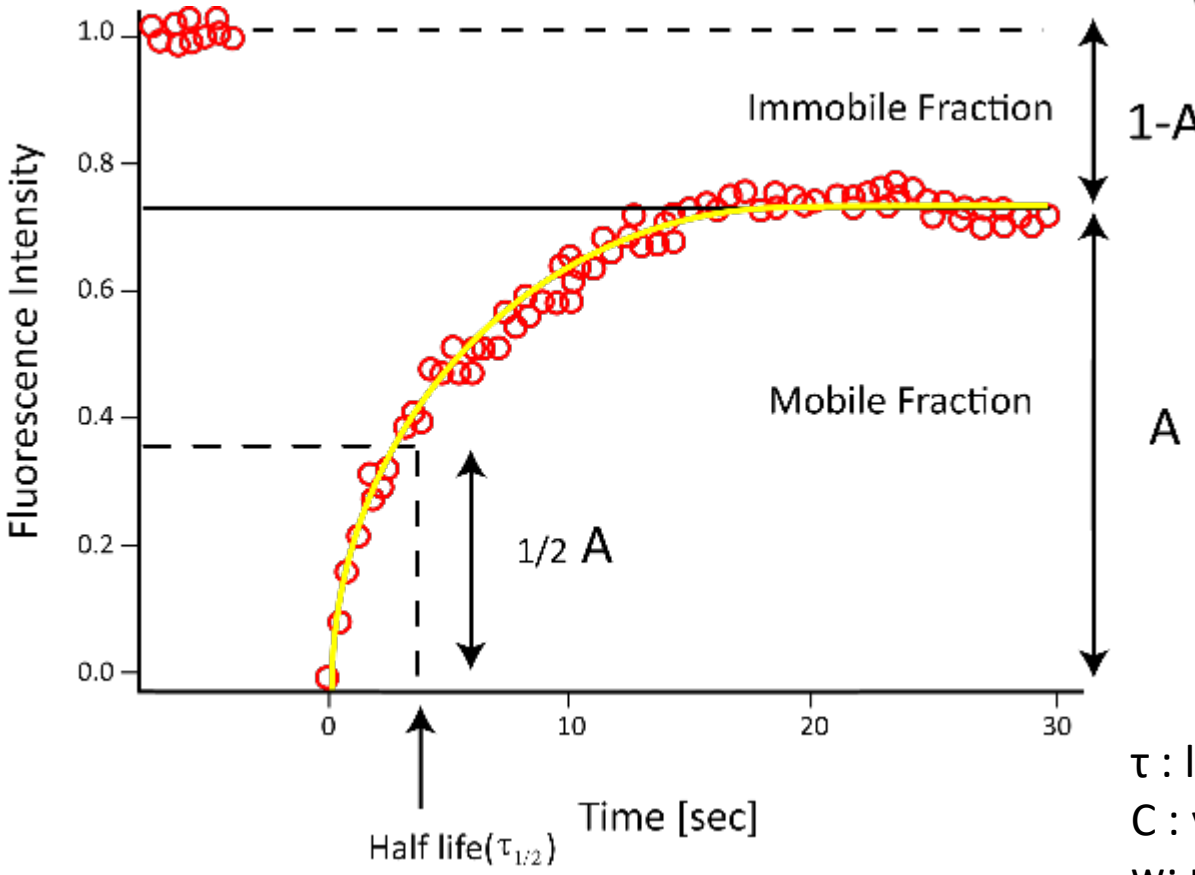
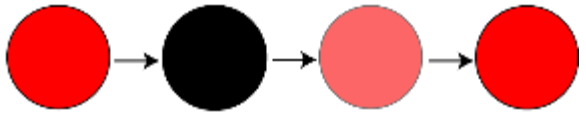
FRAP Curve and equations





$$F_N(t) = A\left(1 - e^{-t/\tau}\right) + C$$

$$\tau_{1/2} = \tau \times \ln(2)$$

$$D_{app} = \frac{0.88w^2}{4\tau_{1/2}}$$

τ : lifetime

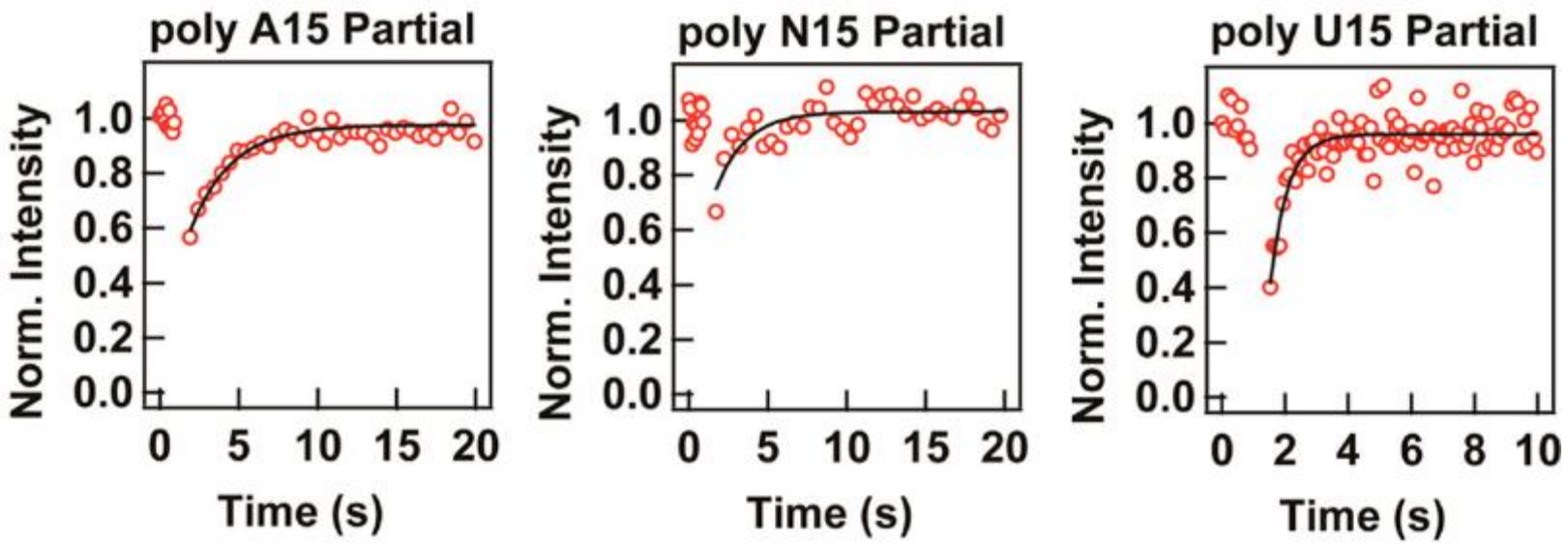
C : y-intercept of the recovery curve

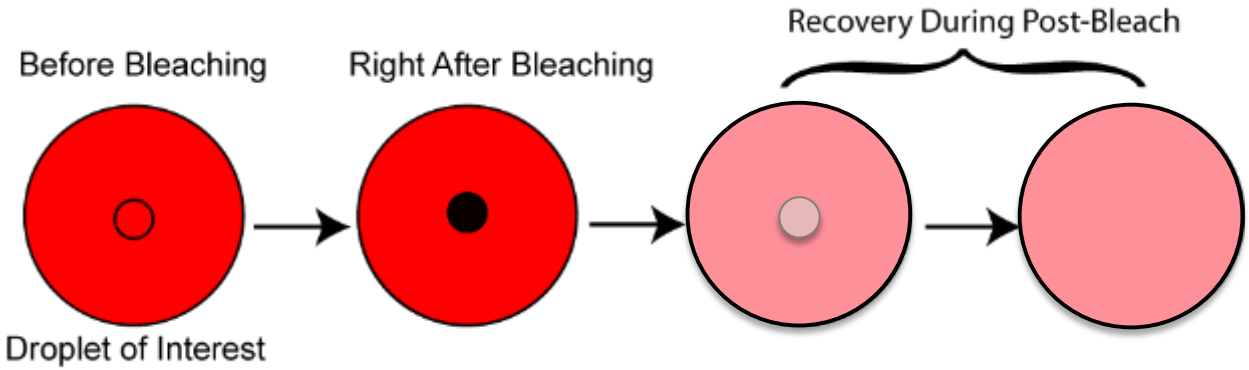
w: radius of the bleached area

Halflife of recovery is the time where fluorescence has recovered to half of its maximum value after bleaching

Axelrod, Daniel, et al., *Biophysical journal* 16.9 (1976): 1055-1069.

Partial droplet bleaching RNA recovery in polyU/spermine coacervate





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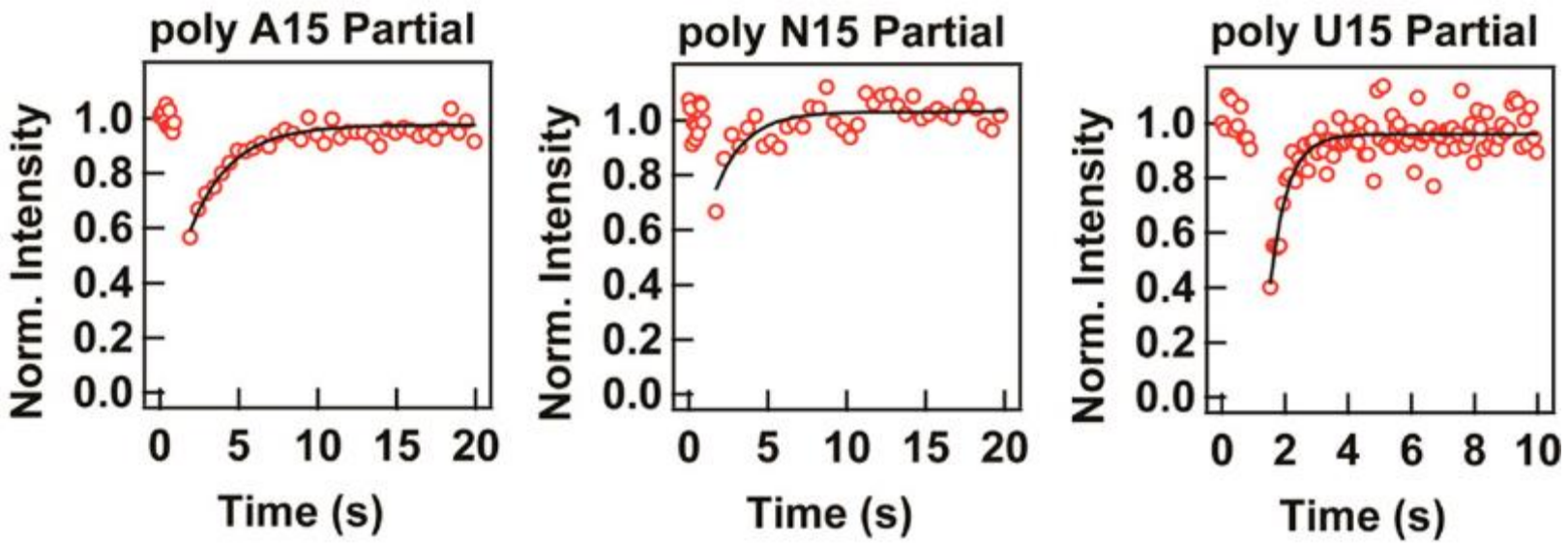

	Poly A15	Poly N15	Poly U15
Partitioning coefficient (K)	2800	62	44
Halftime of recovery, $\tau_{1/2}$ (s)	1.6 ± 0.4	1.6 ± 0.4	0.5 ± 0.2
Apparent Diffusion Coefficient ($\mu m^2 s^{-1}$)	0.8 ± 0.3	0.8 ± 0.2	4 ± 1

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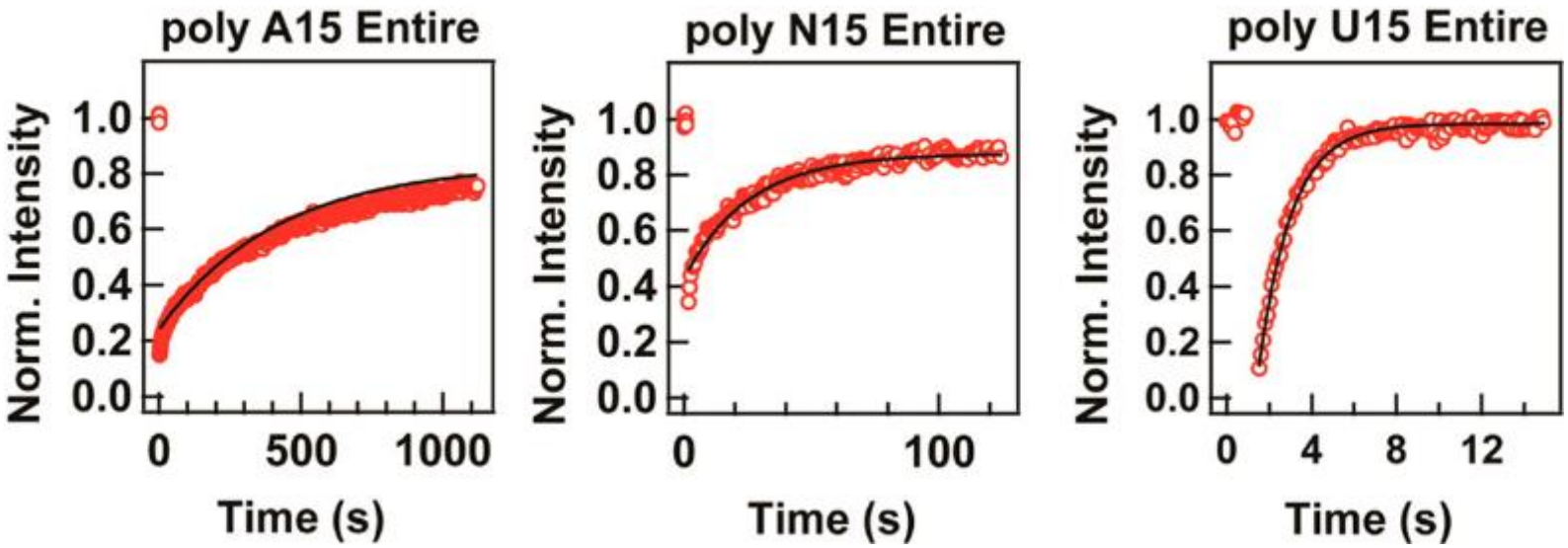
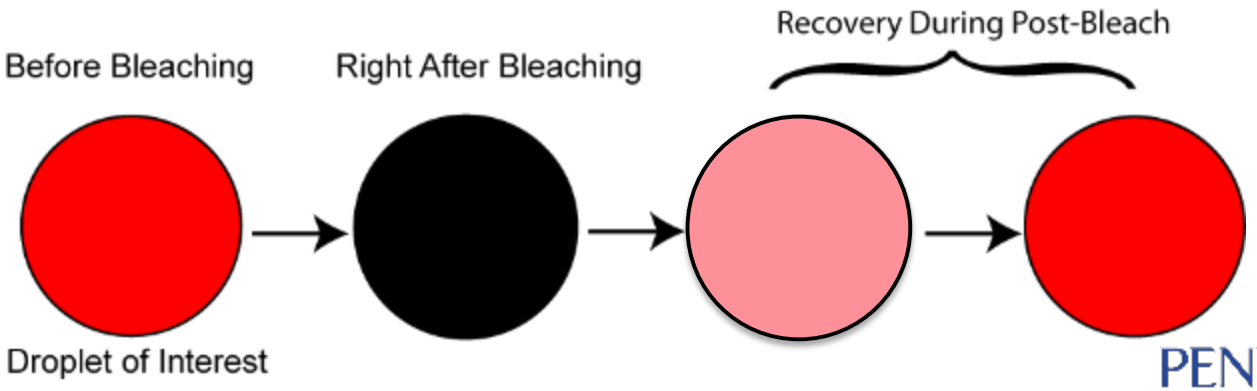
Entire droplet bleaching RNA recovery in polyU/spermine coacervate





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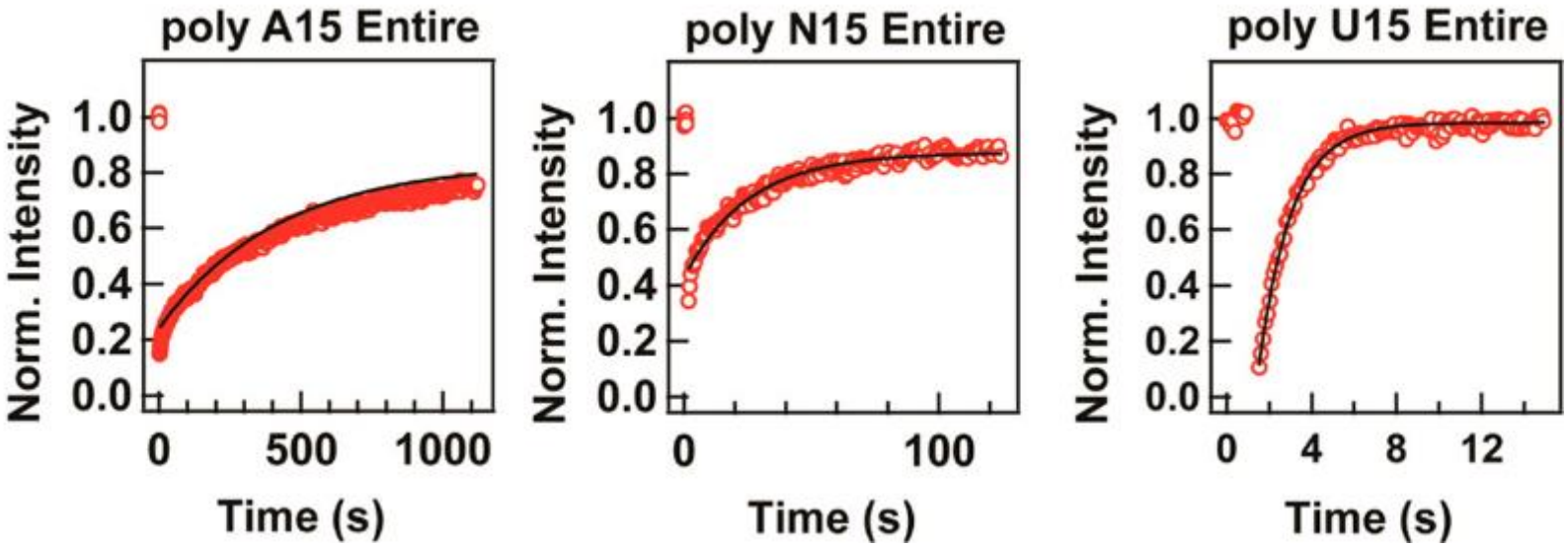
Entire droplet bleaching RNA recovery in polyU/spermine coacervate



	Poly A15	Poly N15	Poly U15
Partitioning coefficient (K)	2800	62	44
Halftime of recovery, $\tau_{1/2}$ (s)	290 ± 20	23 ± 4	1.0 ± 0.3
Apparent Diffusion Coefficient ($\mu m^2 s^{-1}$)	0.005 ± 0.001	0.05 ± 0.01	1.9 ± 0.5

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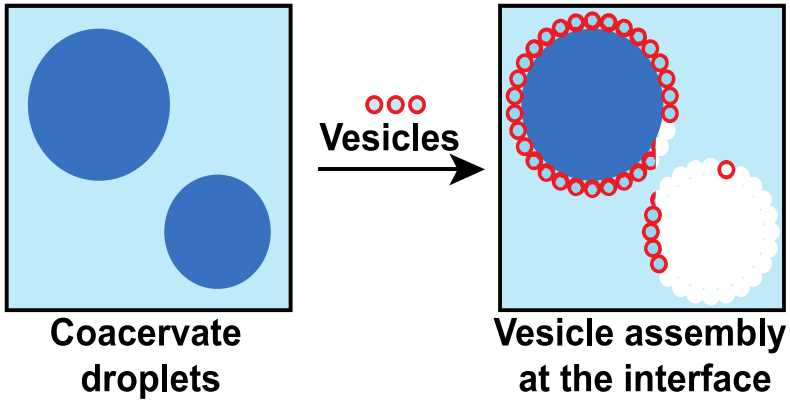
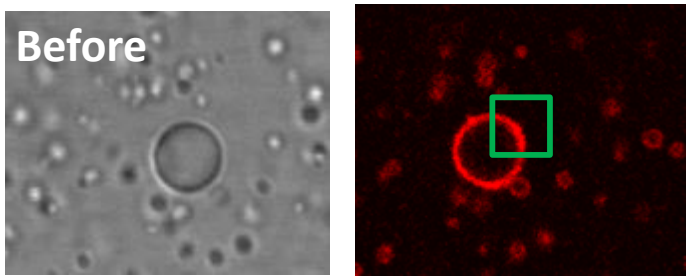
Vesicle assembly around coacervate droplets

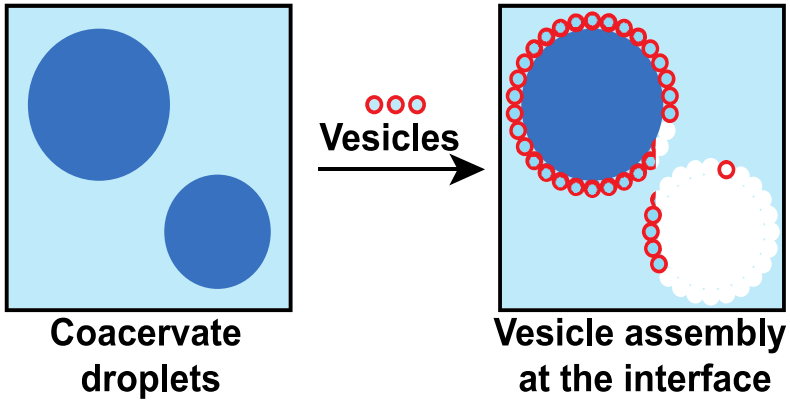


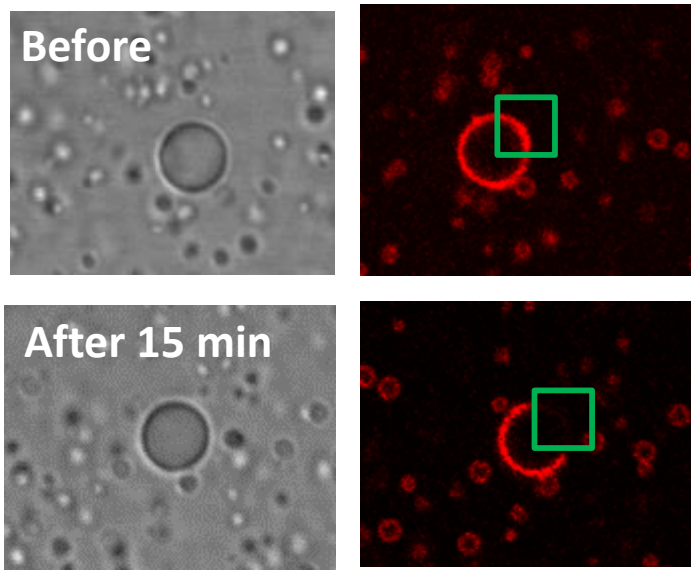


- Vesicles (90 nm) composed of five different phospholipids assembled at the interface in polyU/spermine coacervate system

- The PEGylated lipid was added to prevent the negatively charged vesicles from aggregating due to excess polycation (spermine)

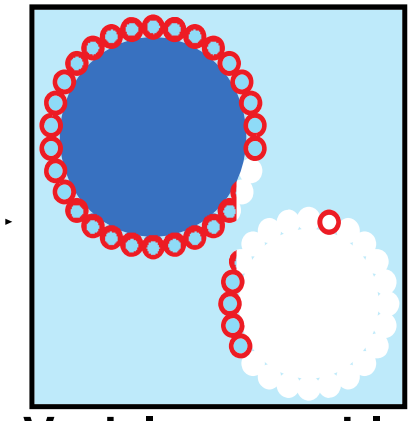
Vesicle assembly around coacervate droplets

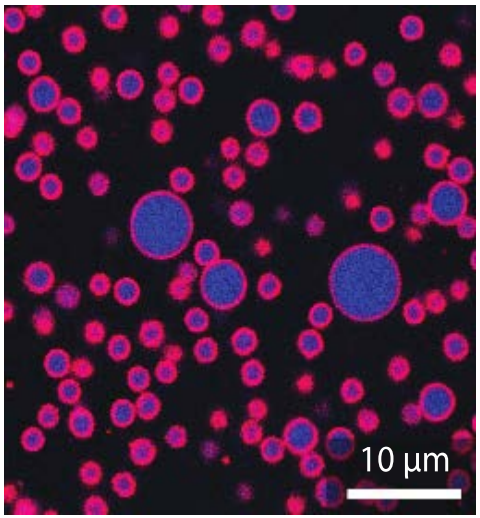


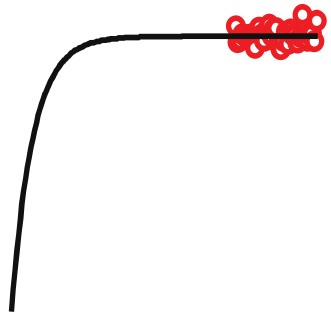


- FRAP showed no recovery after 15 min. indicating liposomes are immobile.
- DLS confirmed no lipid bilayer formation and vesicles remain same size.

RNA diffusion in vesicle coated coacervate droplets



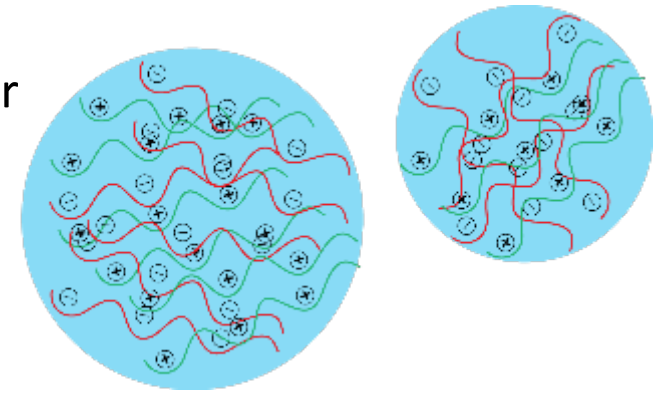




RNA diffusion into/out of the coacervate droplets wasn't impeded.

Conclusions

- Low complexity RNA and simple polyamines were used to form coacervates.
- Rapid exchange of oligonucleotides observed for partial droplet bleaching
- Entire droplet recovery depended on binding interactions and the supernatant phase concentration
- Interfacial accumulation of lipid vesicles did not inhibit exchange of biomolecules



Aumiller Jr, W. M., **Pir Cakmak, F.,** Davis, B. W., & Keating, C. D., *Langmuir*, *32*(39), 10042-10053.

Thank you!

Acknowledgments

Keating Group

- Dr. Christine Keating
- Dr. William Aumiller
- Dr. Brad Davis

Funding:

Exobiology Program

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