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MODERN PARTICLE CHARACTERIZATION TECHNIQUE SERIES IV: MULTI-LASER NANOPARTICLE TRACKING ANALYSIS (NTA)

Sean Travers, PhD

Outline of topics

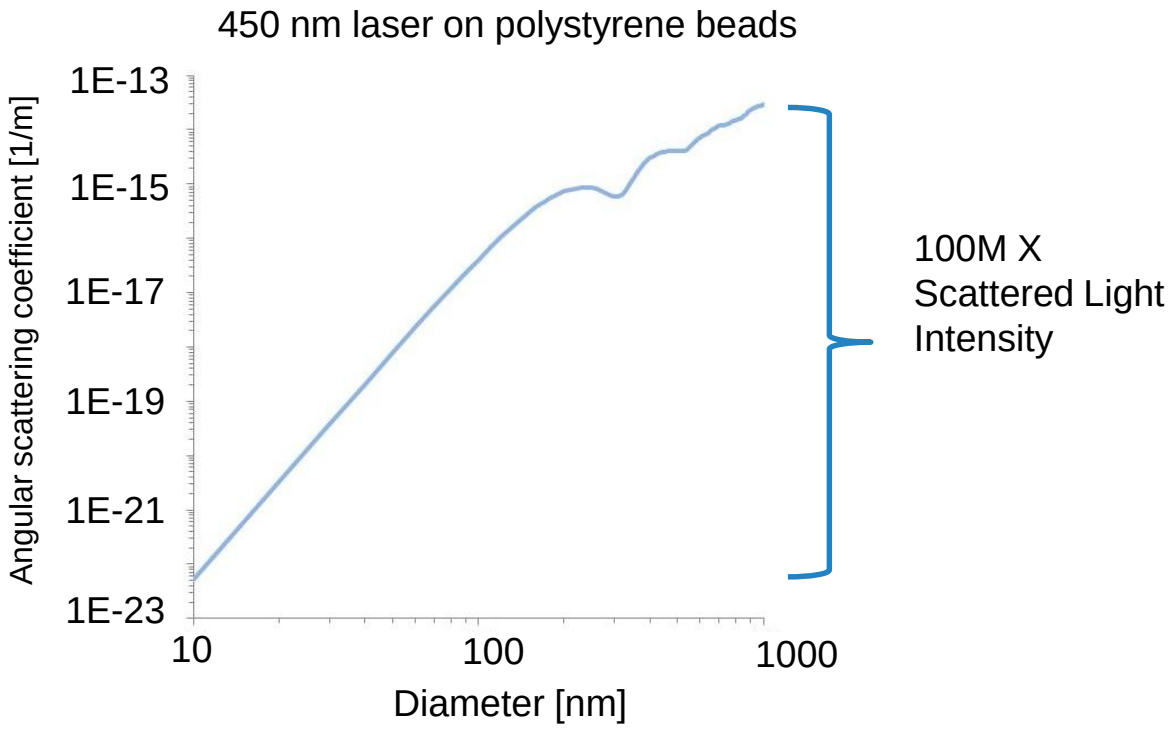
- What is NTA?
- What makes the ViewSizer unique?
- Biotech Application Virus
- Production in Gene Therapy
- Infectious Titer
- Quality Control
- Stability testing
- Extracellular Vesicles, Exosomes
- Liposomal Adjuvants
- Wrap-up/Conclusions
- Questions?

Unmet Needs in Particle Sizing

- Visualization of polydispersed nanoparticles
- Accurate & reproducible measurement of:
  - Particle number concentration
  - Particle size distribution
  - Particle kinetic processes
  - In this talk the focus will be on the first two applications

Problem

- When looking at a polydisperse samples, the scattered light varies drastically when going from the low nm range up to a micron
- Prevents accurate tracking, sizing and analysis of samples that contain particles of varying sizes
- Unfortunately, that happens in a fair number of samples of interest, especially in the biotechnology world!



Problem is Well Known

INTERNATIONAL STANDARD **ISO 19430**

Particle size analysis — Particle tracking analysis (PTA) method

"Sample polydispersity affects the ability to track and therefore analyse different size fractions in the particle number-size distribution. … In a polydisperse sample large particles scatter a lot more than small particles making it difficult to detect or track small size particles."

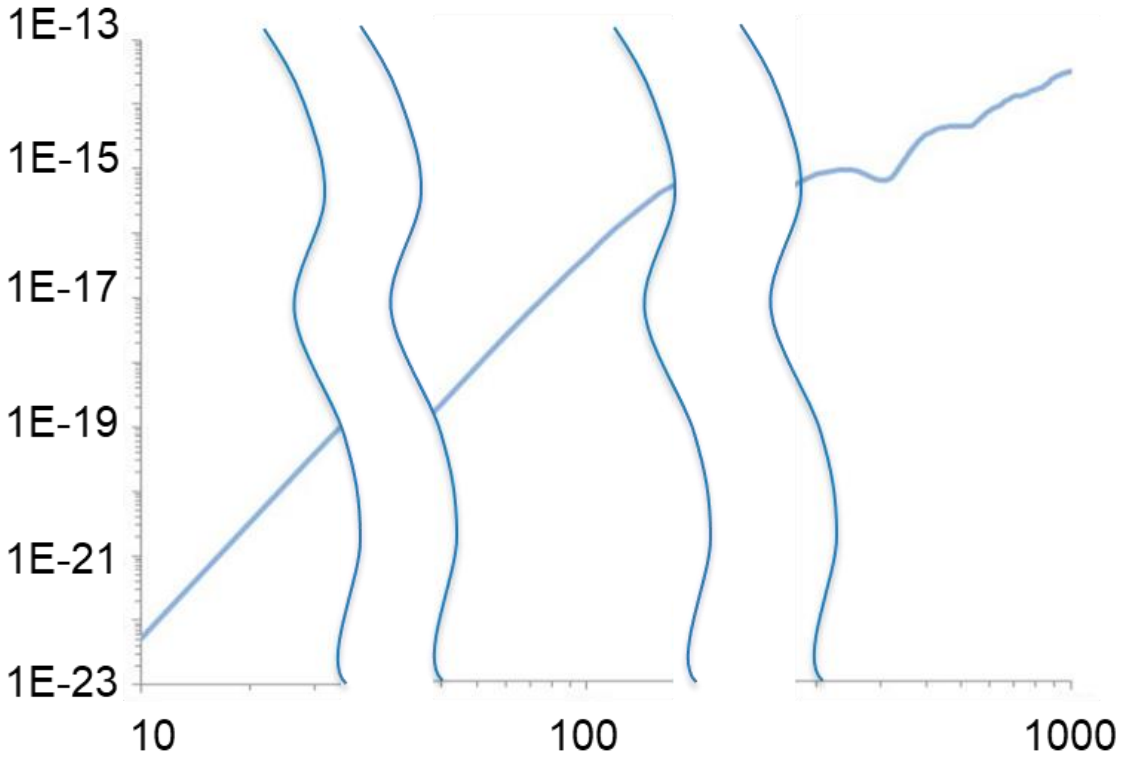
Reference Customer

Dr. Sadik Esener
Director, OHSU's Center for Early Cancer Detection Research

"In our tests leading up to the purchase of our ViewSizer 3000, **we confirmed this easy to use bench top instrument meets all our needs for visualizing, sizing and counting nanoparticles** such as live viruses, exosomes, silver, RNA, and YAG"

"The ViewSizer is **the first product we've found that can effectively characterize particles in polydisperse samples** and its unmatched visualization of all particles, even in complex samples, removes elements of mystery associated with other methods."

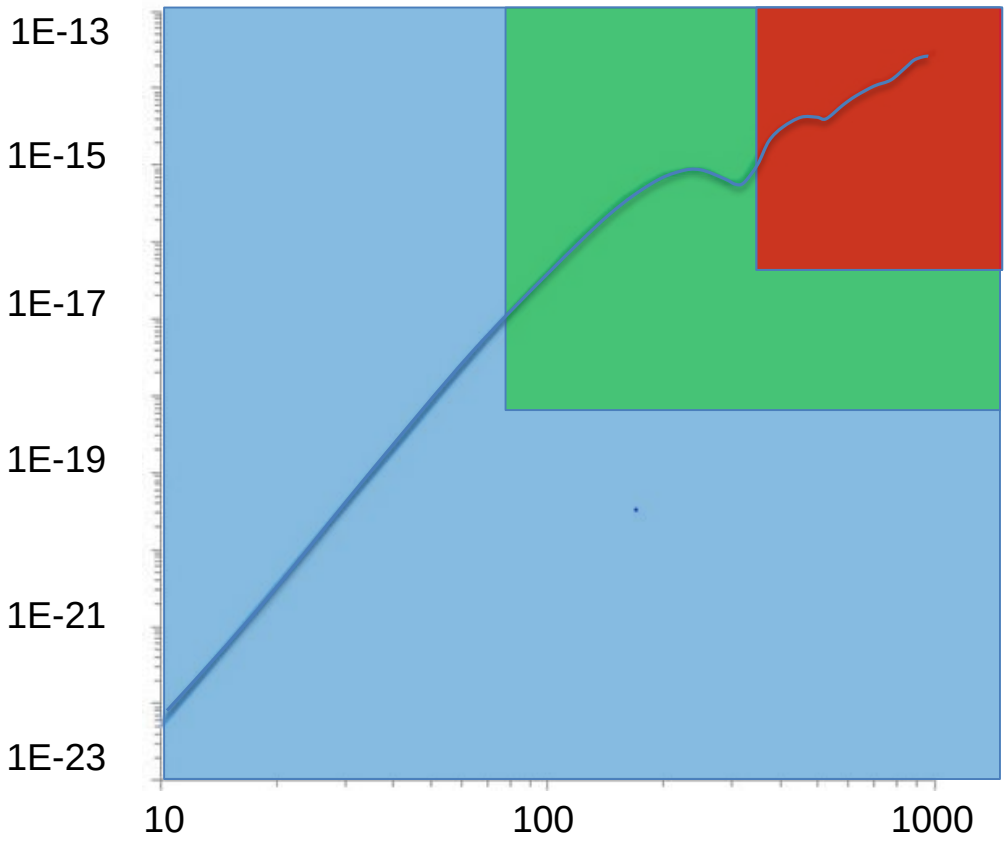
The Solution



Break the problem into manageable segments

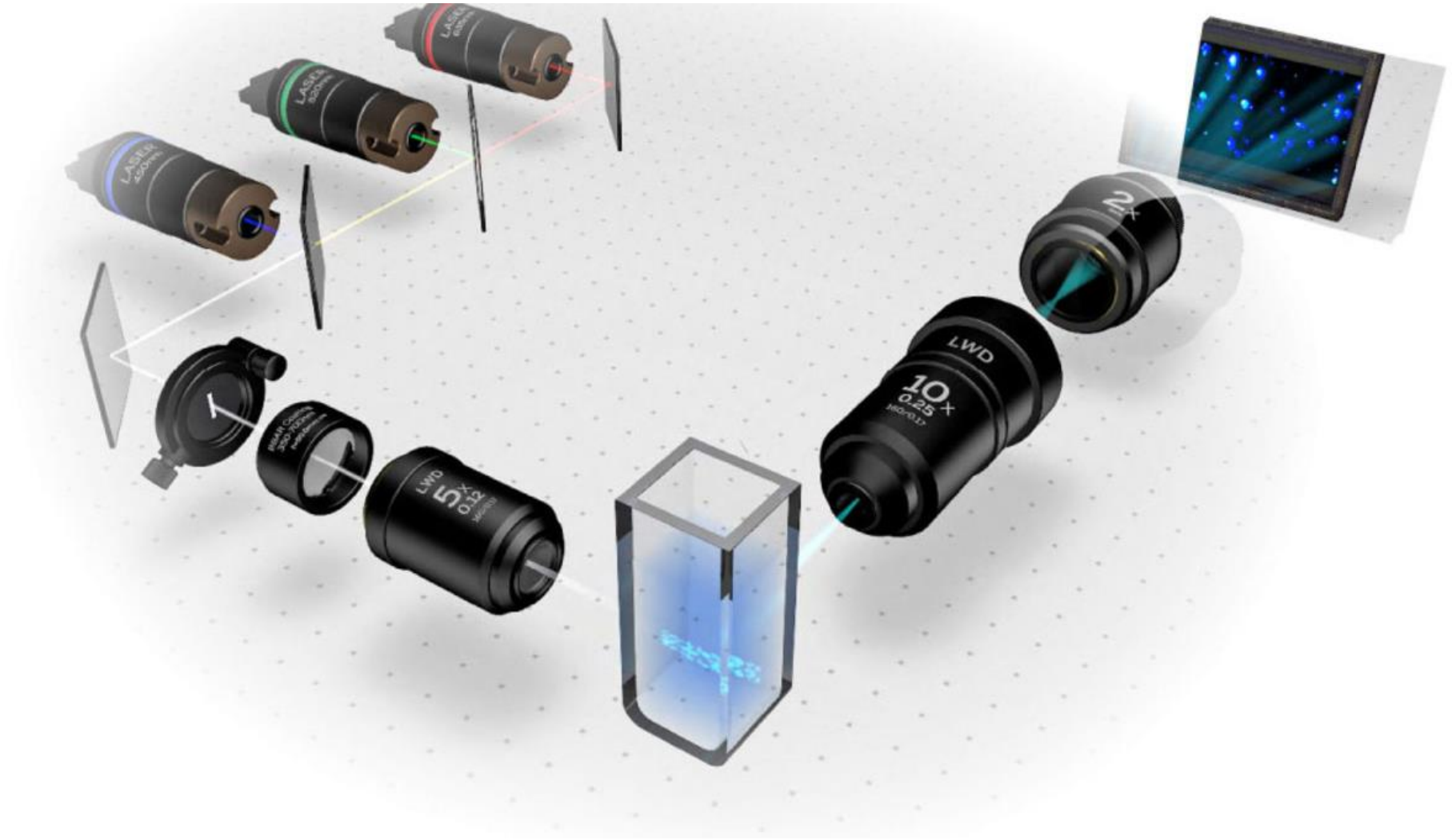
The ViewSizer Solution

(Multispectral Advanced Nanoparticle Tracking Analysis – MANTA)



Simultaneous Multispectral Particle Analysis

Internal Components



Pretty Little Package

Crucial Adaptations to Sample Presentation

- In order to get representative sampling on average we typically record 25 videos per sample measurement, our sample device is shown at right.
  - Pretty standard cuvette, slits are designed to allow the light sheet to enter
  - Measure (via video) light scattered by the particles at 90 degrees
  - Crucially we have a very tiny stir bar at the bottom of the cuvette
  - Allows us to properly mix in between measurements, presents a fresh aliquot of particles for each recording
  - This avoid manually moving the sample and interfering with Brownian motion
  - Removable insert means easy cleaning
  - Typical lab uses multiple cells

Cuvette & Insert

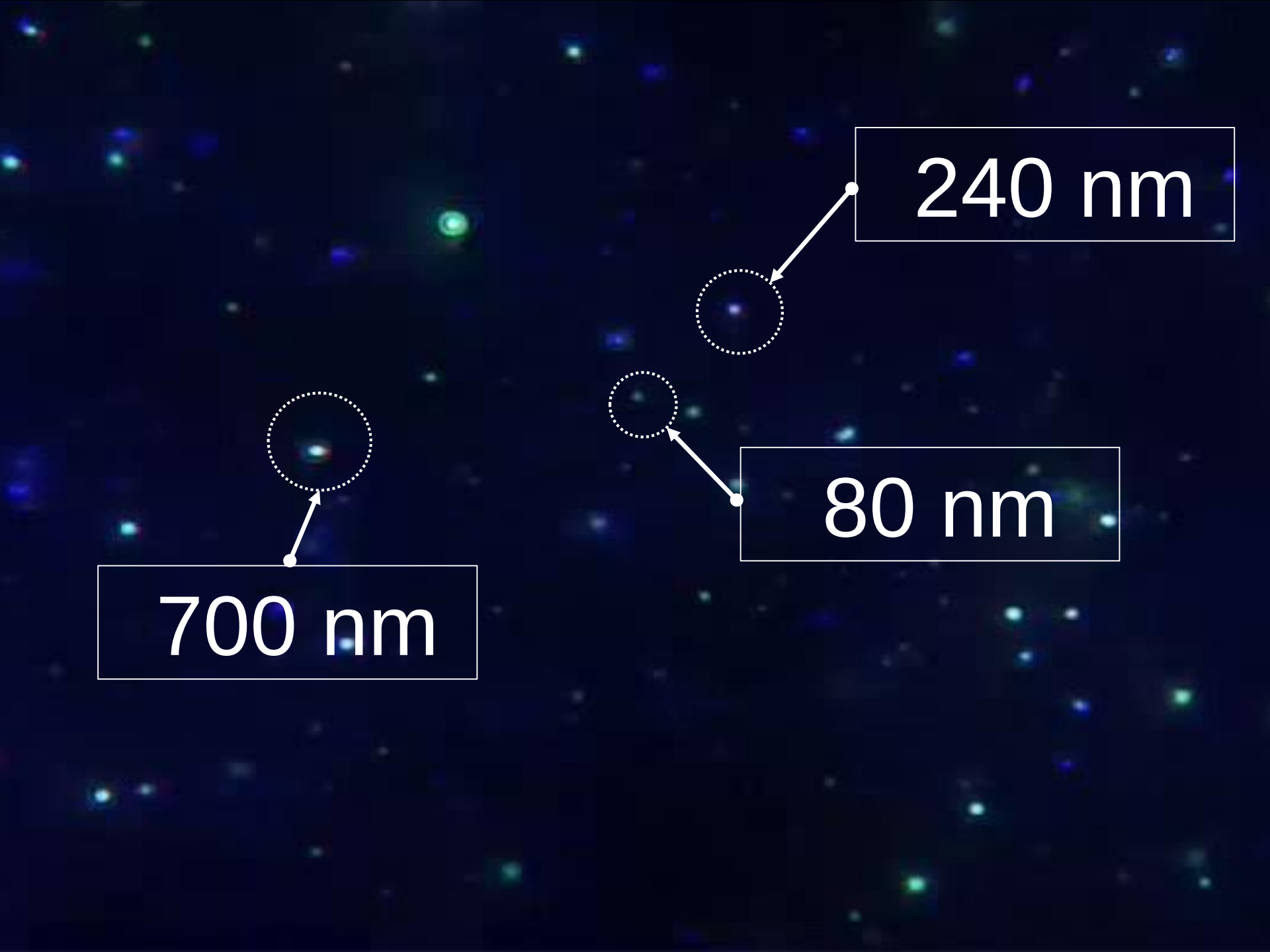
Everyone's favorite part - the math!

- Using this lovely equation we are able to back calculate the diameter of our particle from how far it moves during Brownian motion
- Trick is doing this with a many particles and using the proper laser for the size range of the particle of interest
- Requires a lot of CPU processing power (custom build a tower for the instrument)
- In order to prevent missing out on particles, we record our videos in one session, and then process the data in a second session
  - Prevents us from dropping frames and missing out on crucial particles within your sample!

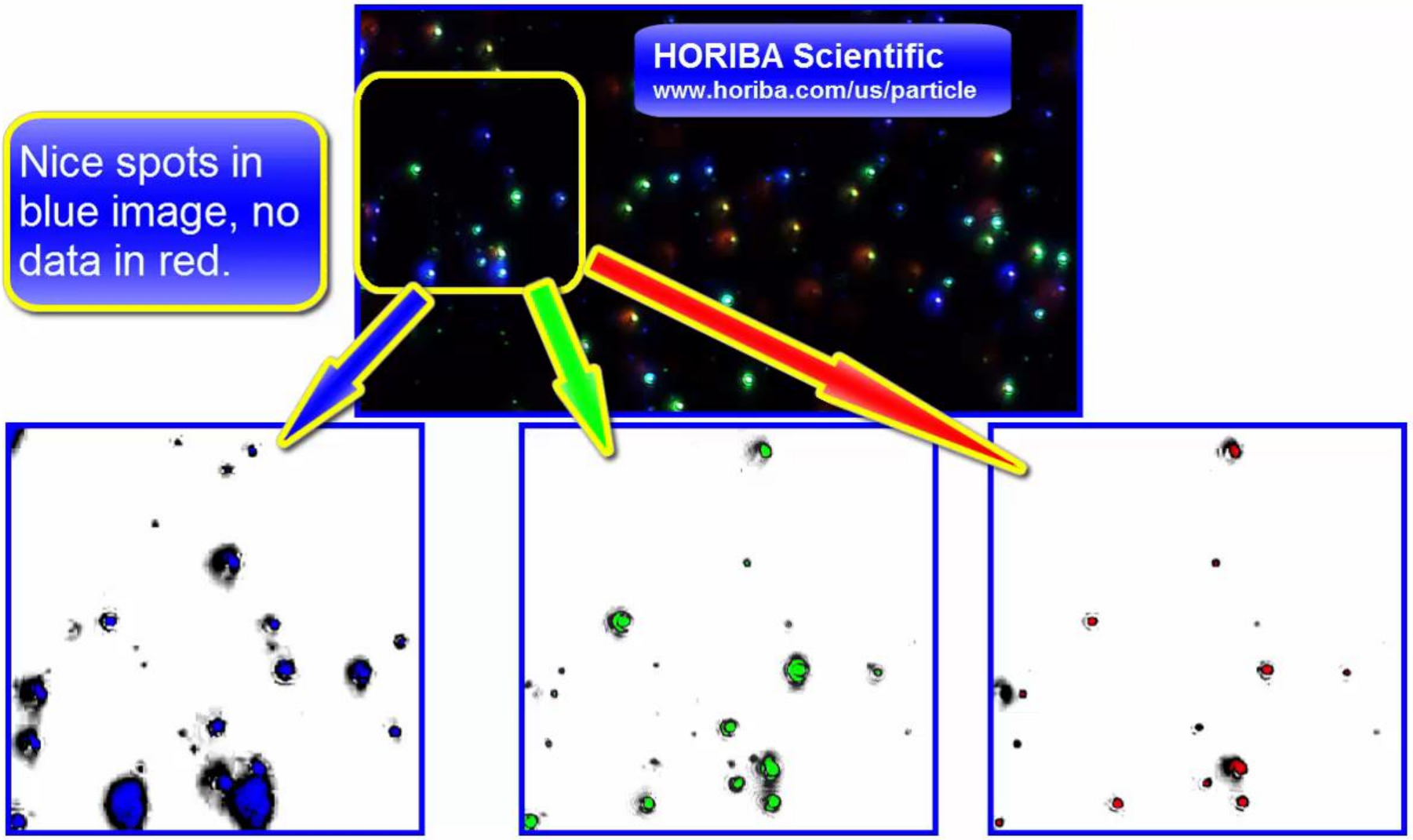
Stokes-Einstein Equation

$$d_H = \frac{kT}{3\pi\eta D}$$

d_H = hydrodynamic diameter (m)
k = Boltzmann constant (J/K=kg·m2/s2·K)
T = temperature (K)
η = solvent viscosity (kg/m·s)
D = diffusion coefficient (m^2/s)

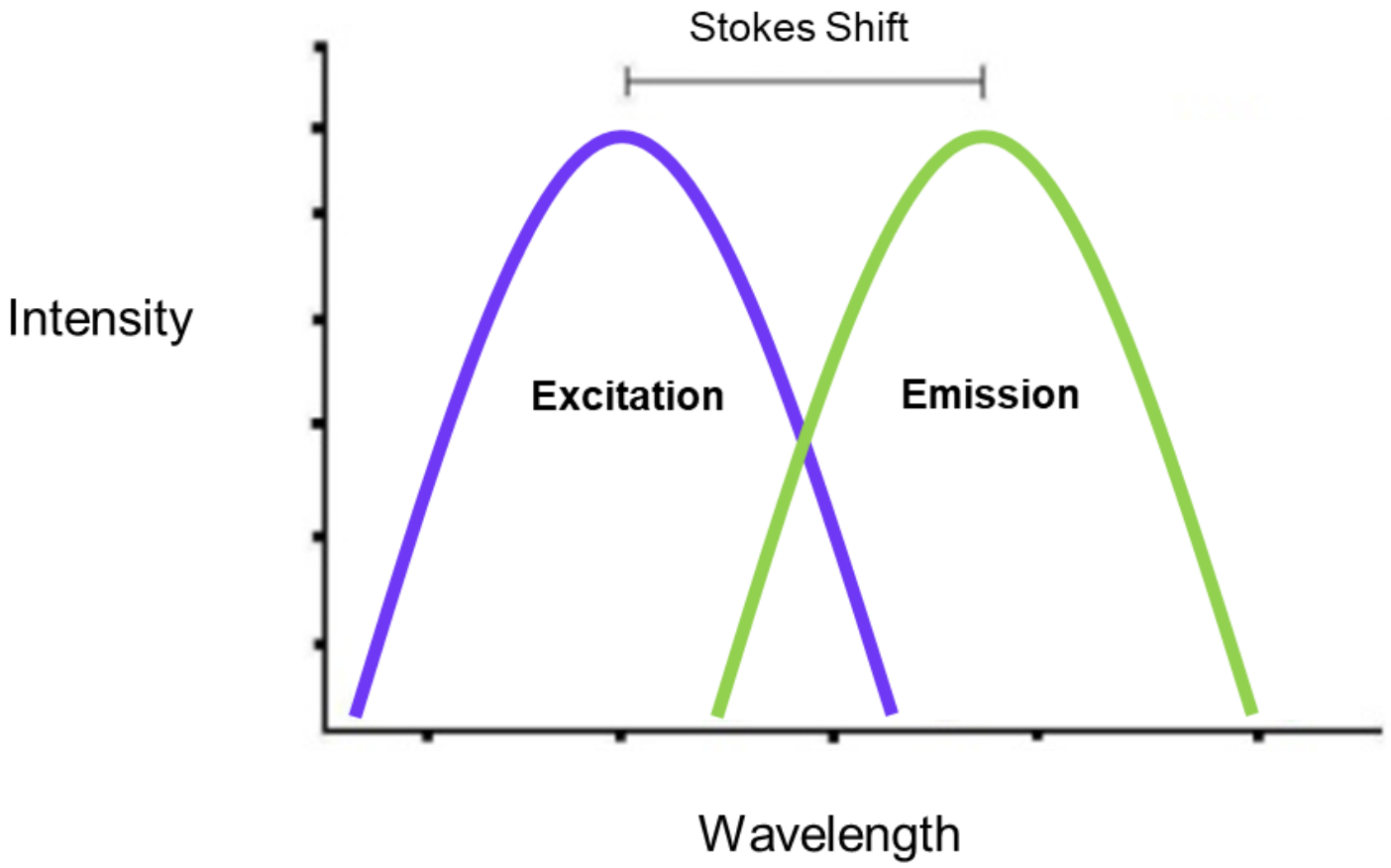

240 nm
80 nm
700 nm

Why three colors?



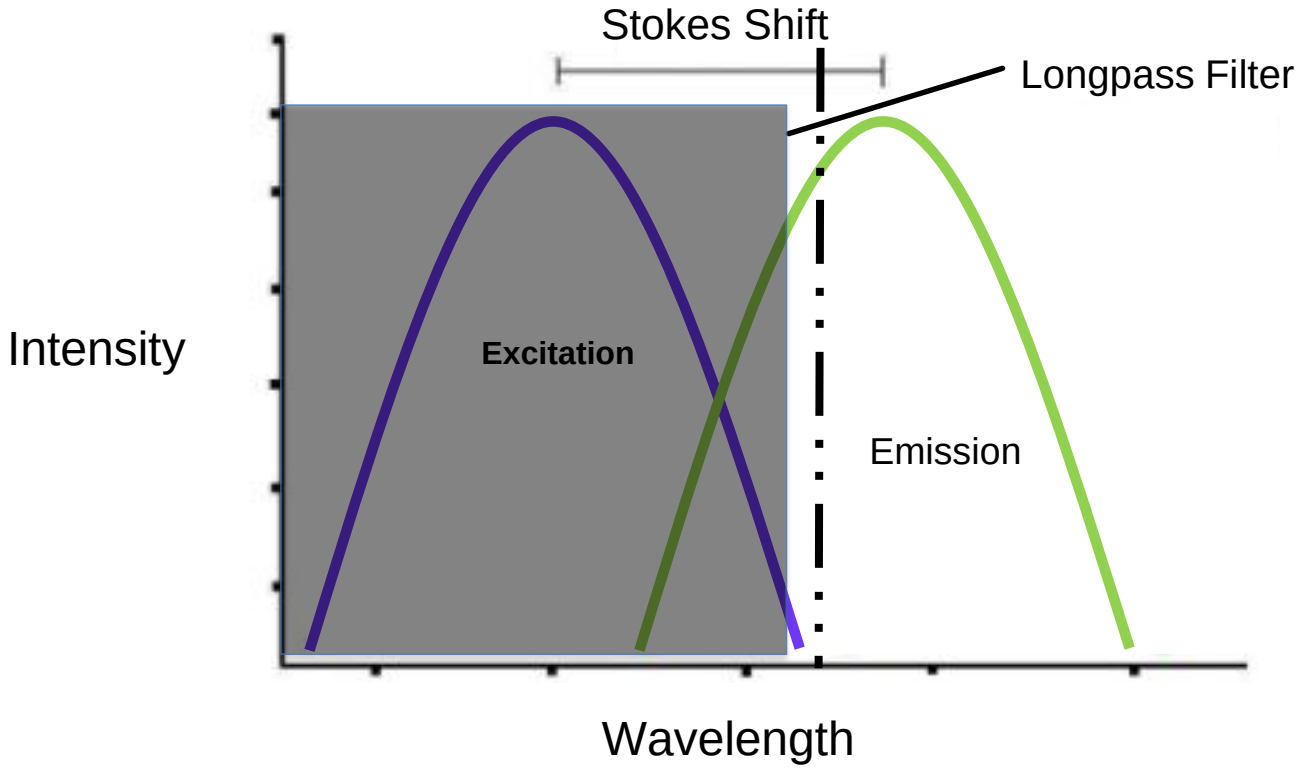
ViewSizer 3000 Fluorescence – Basics

The Stokes shift



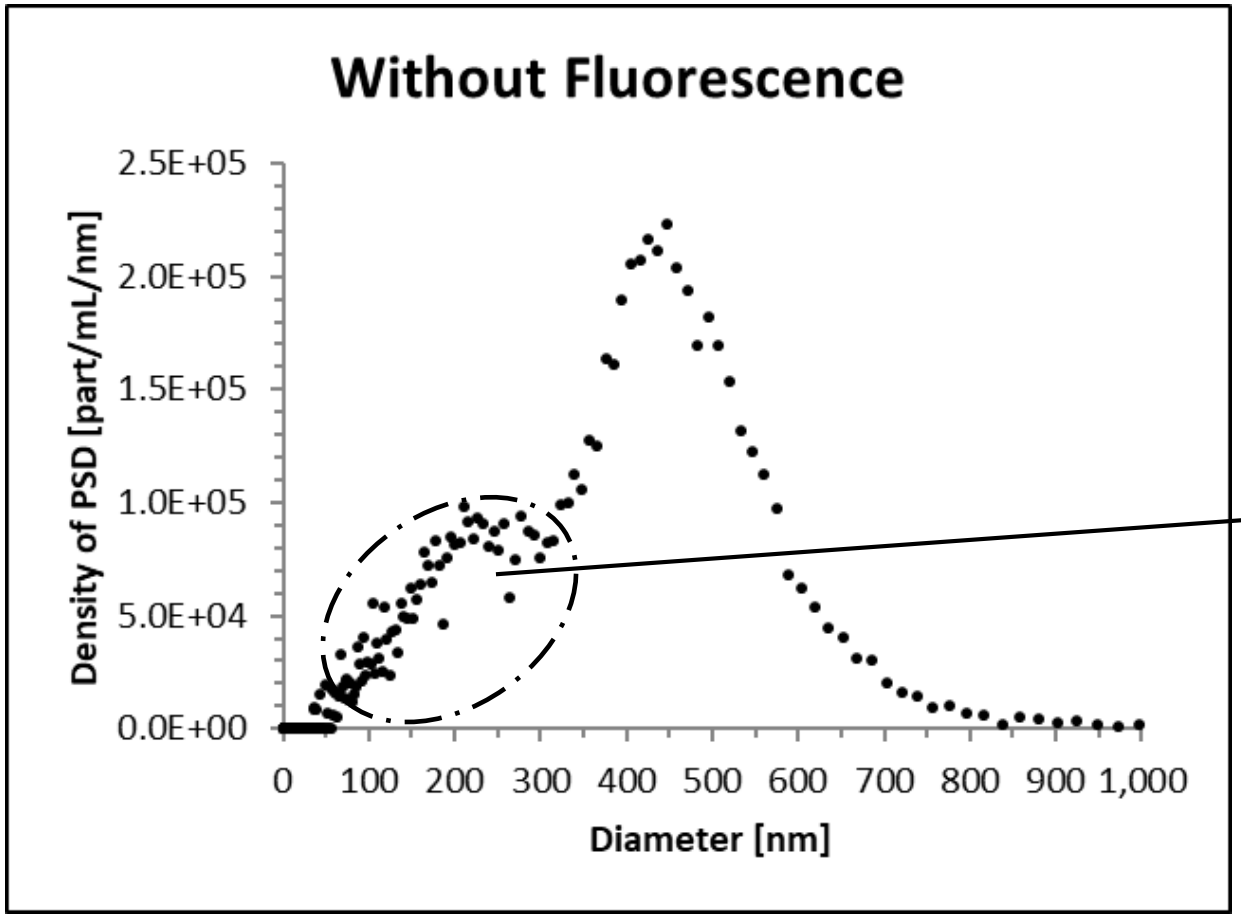
ViewSizer 3000 Fluorescence – Basics

Filter enables detection of only fluorescent signal



ViewSizer 3000 Fluorescence Results

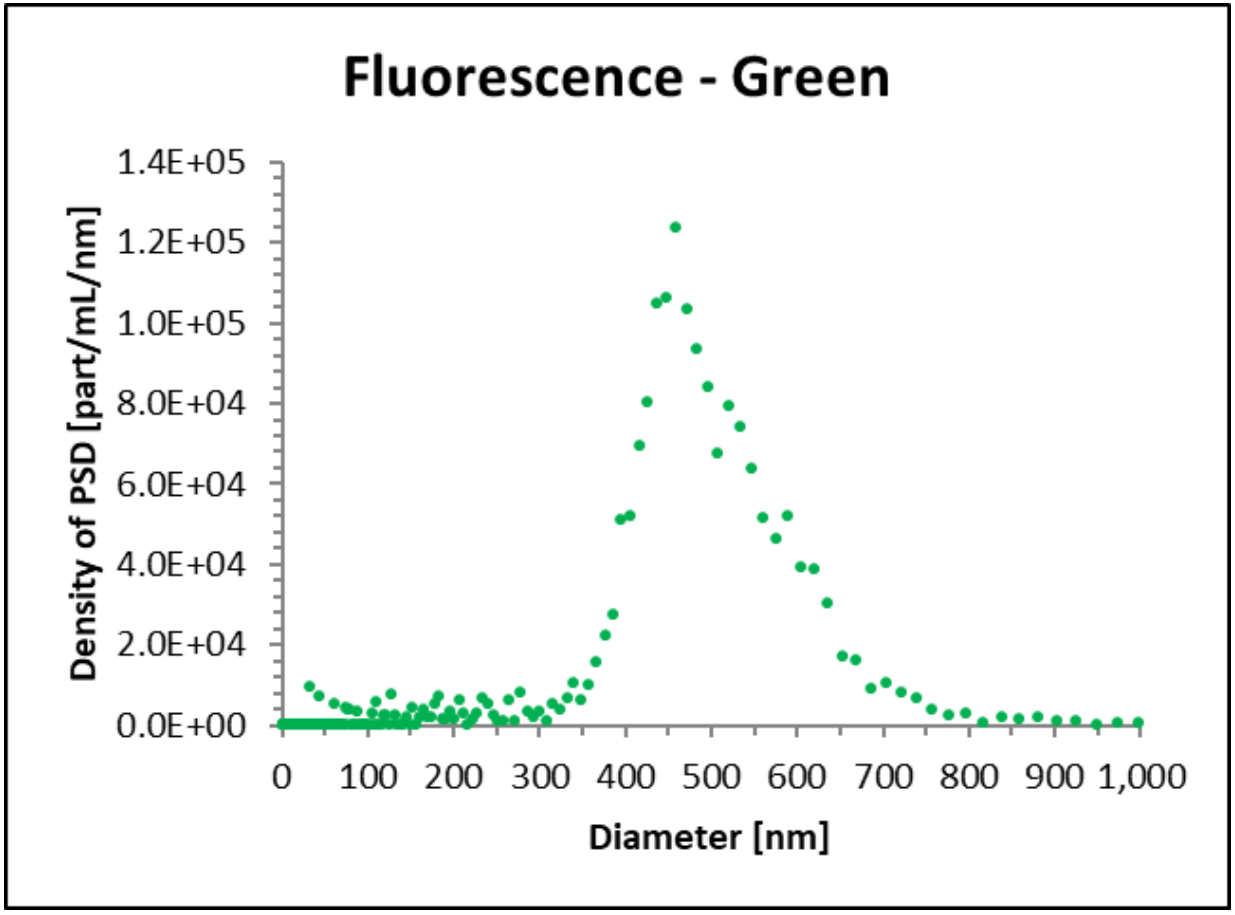
Mix of Three Carboxylate Fluorescent Beads (nominally 500 nm)



Background from diluent

ViewSizer 3000 Fluorescence Results

Mix of Three Carboxylate Fluorescent Beads (nominally 500 nm)



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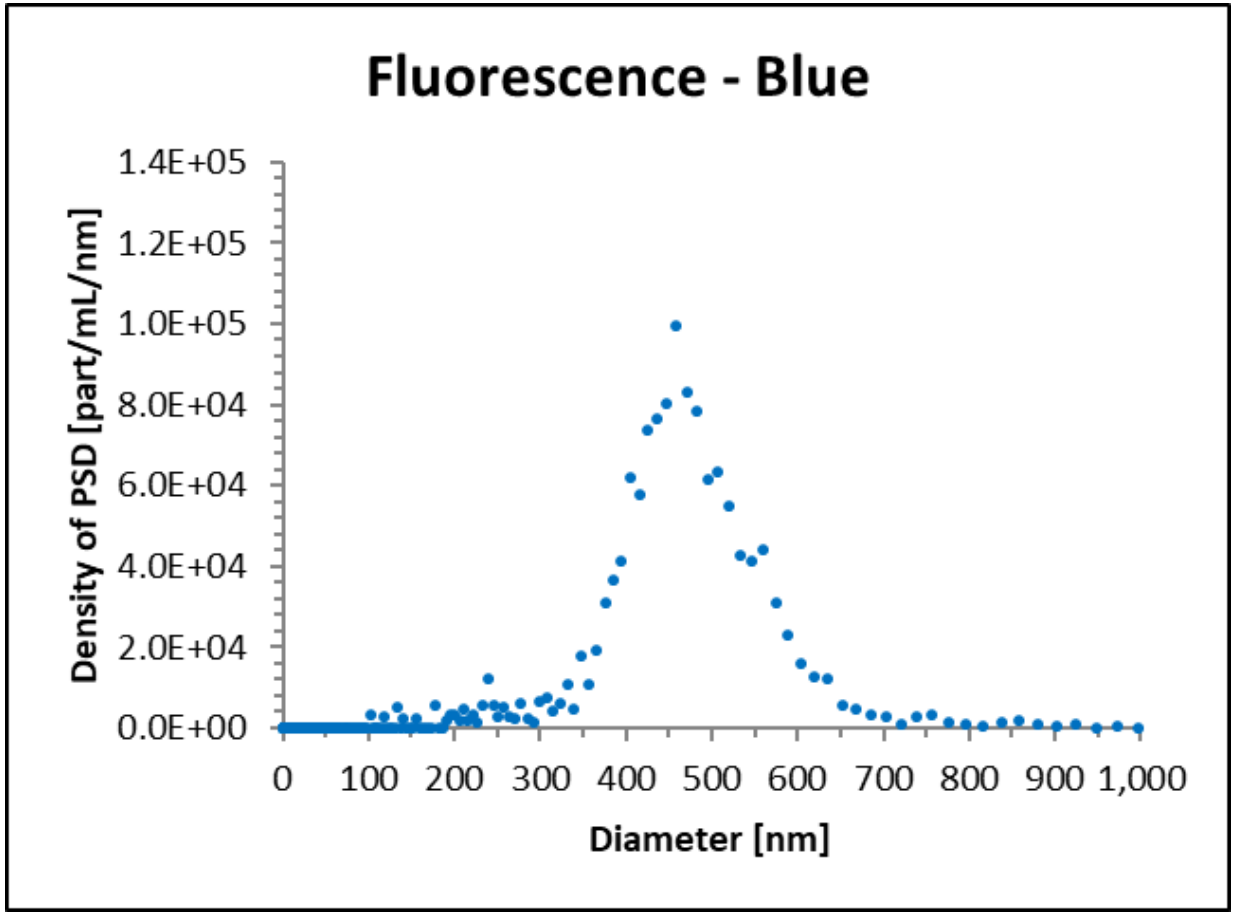

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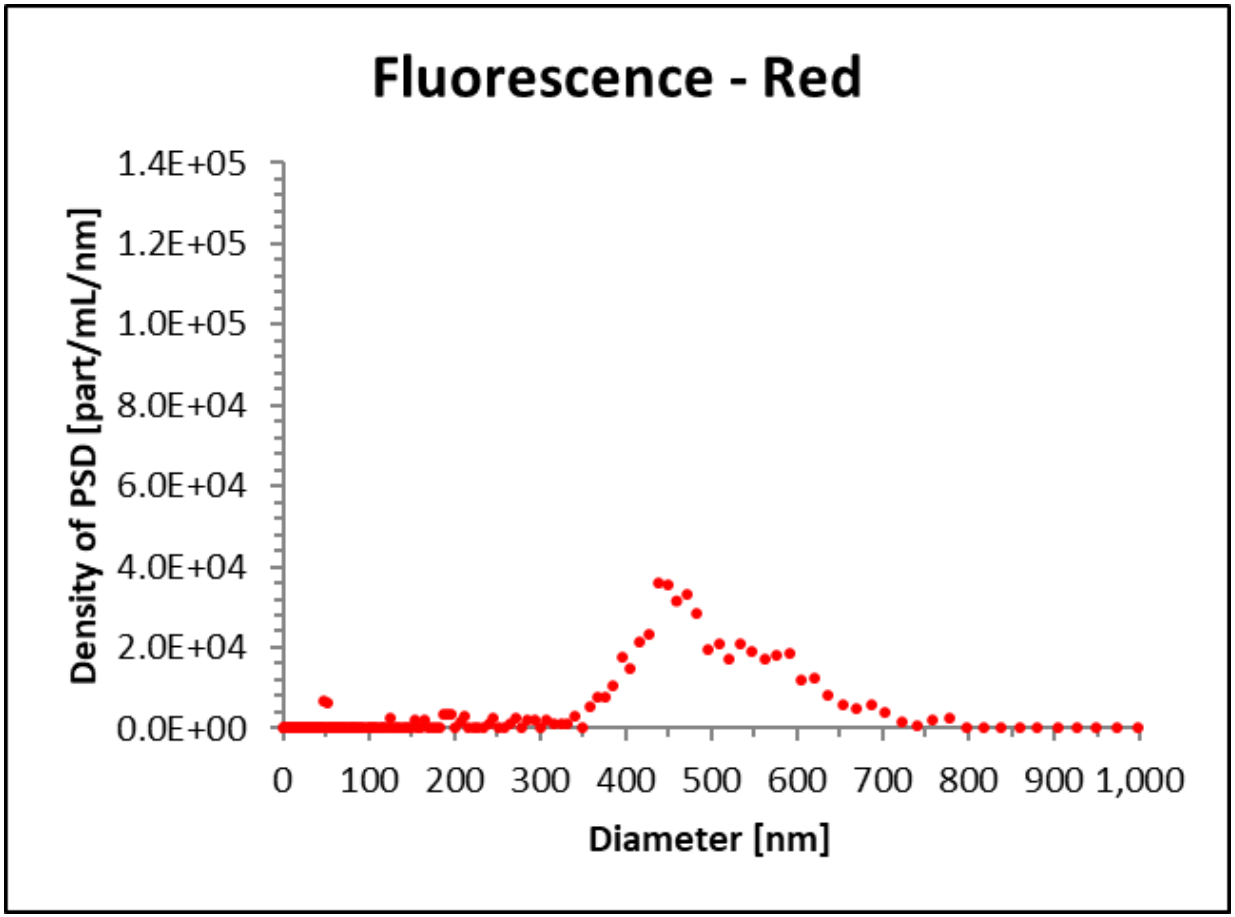

ViewSizer 3000 Fluorescence Results

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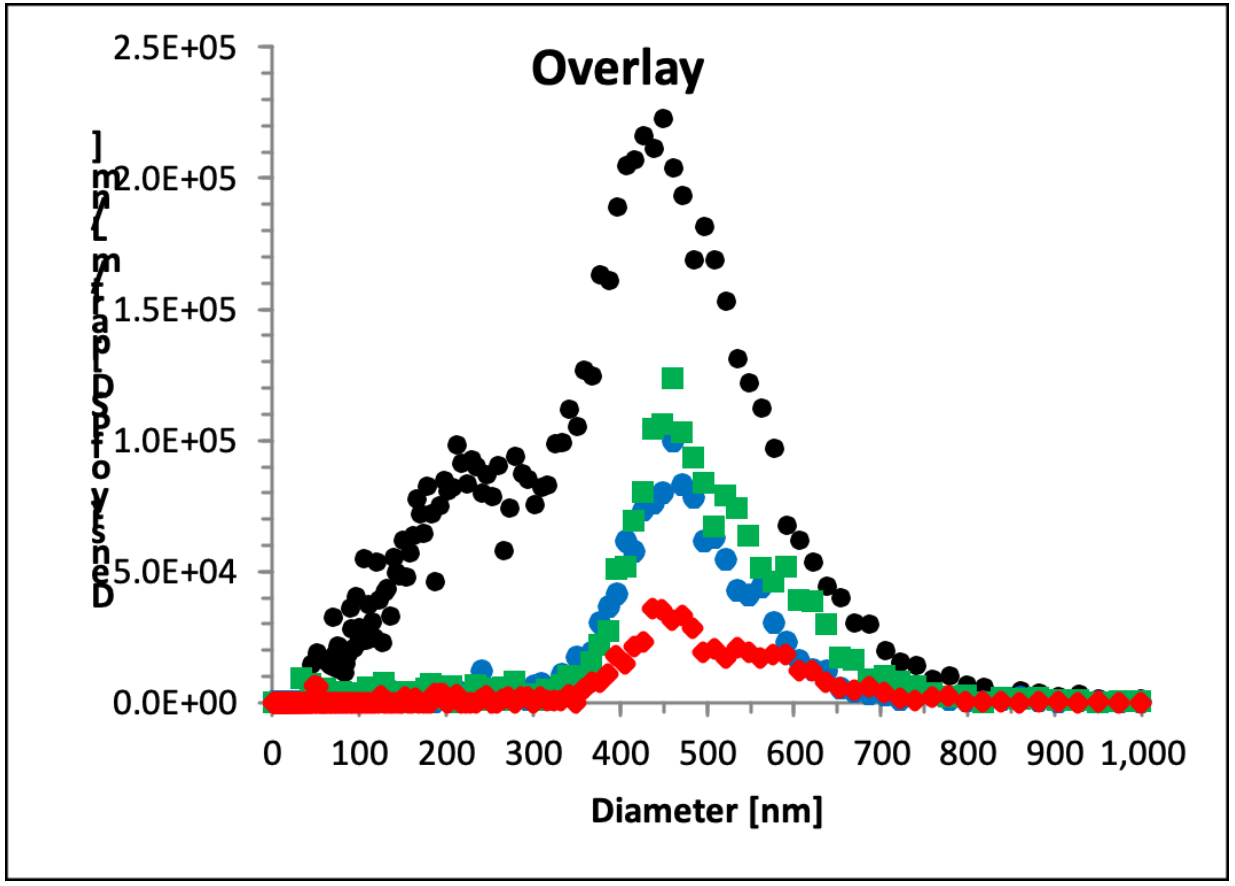
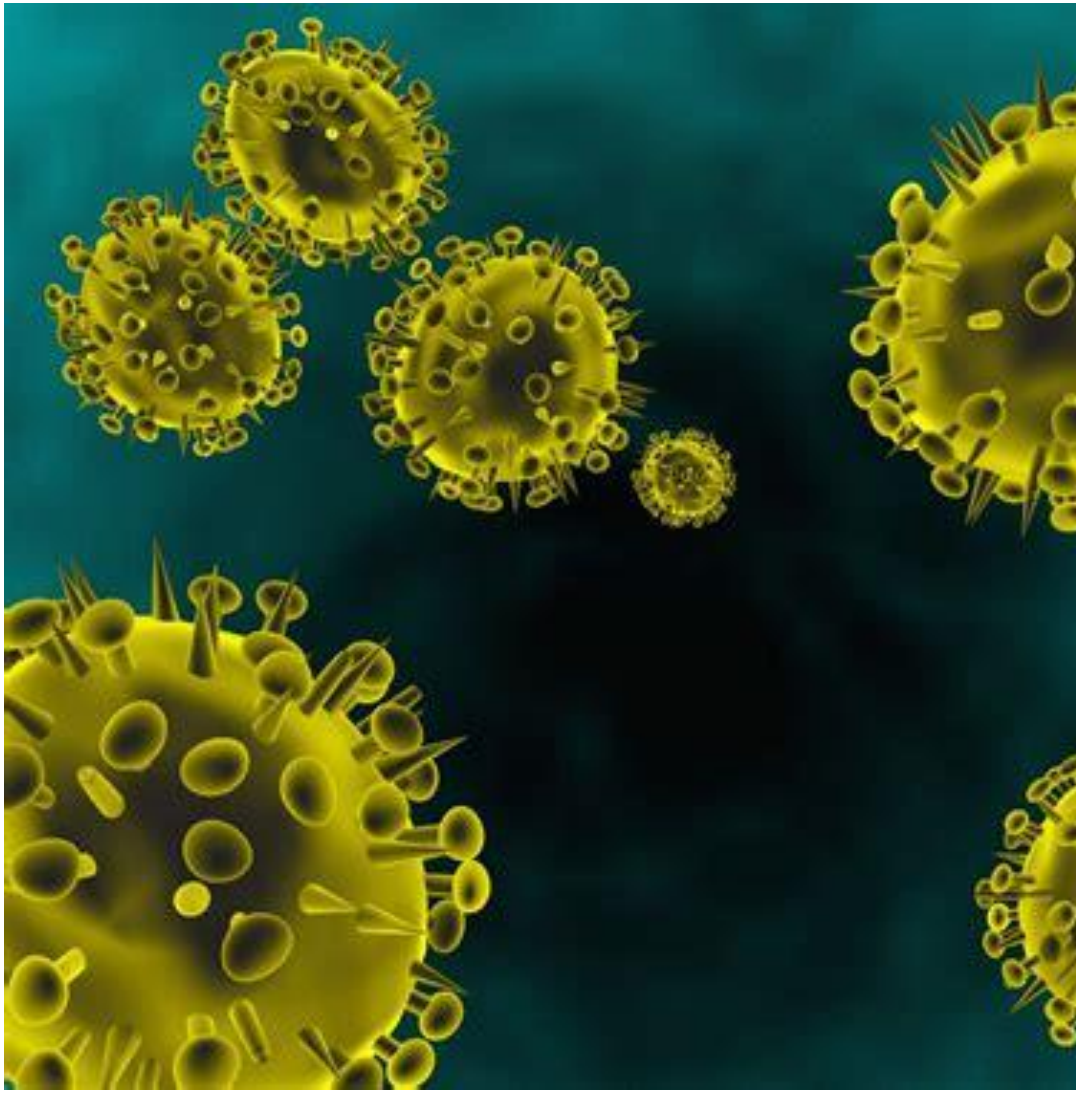


Current Collaborators In Eastern US

- Currently working with two companies in Southern Maryland (NDAs Prevent name sharing)
- Walter Reed Army Institute Research
- UMD - Baltimore Medical School with Niaz Khan

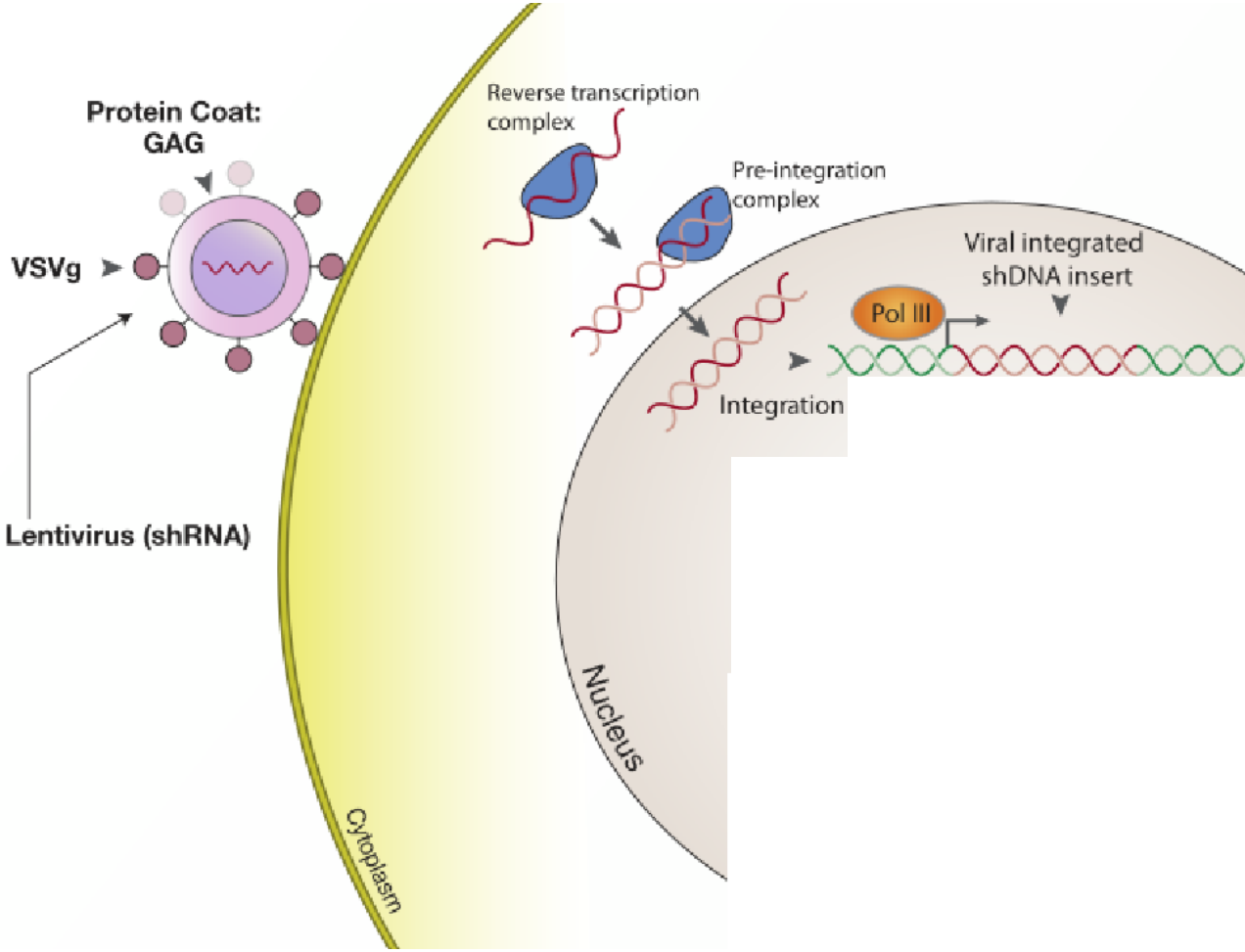
What is a Lentivirus?

- Lente – Latin for slow; meaning long incubation times
- Best known of the Lenti-family is certainly HIV and its various phenotypes (FIV, EIV, BIV, etc.)
- Have the capability to introduce large amounts of viral RNA
  - Capable of infecting a host cell
  - Even non-dividing cells
  - Can create long-term stable cells with consistent gene expression



How?

- Virus binds to exterior of cell
- Viral RNA is inserted (catered to the targeted disease)
- Machinery of the cell creates a complimentary dual-stranded complex
- Migrates into the nucleus where it is inserted into host cells DNA and affects desired changes



Why Lentivirus?

- Slower incubation times, and the creation of stable non-dividing cells have shown some promise in reducing the immune response associated with gene therapies.
- Major hurdle in treating genetic diseases
- Children's Hospital of Philadelphia (CHOP) - has used an extremely similar process to cure congenital blindness in human patients

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3044498/

How do we fit in?

- Lentiviruses are ~100nm
- They are efficacious as dimers, and possible even as detritus that has RNA attached
- Goals
  - Measure the size of their sample and aggregates
  - Get an accurate count across the size range
  - Which could possibly correspond to their infectious titer assays

Infectious Titer

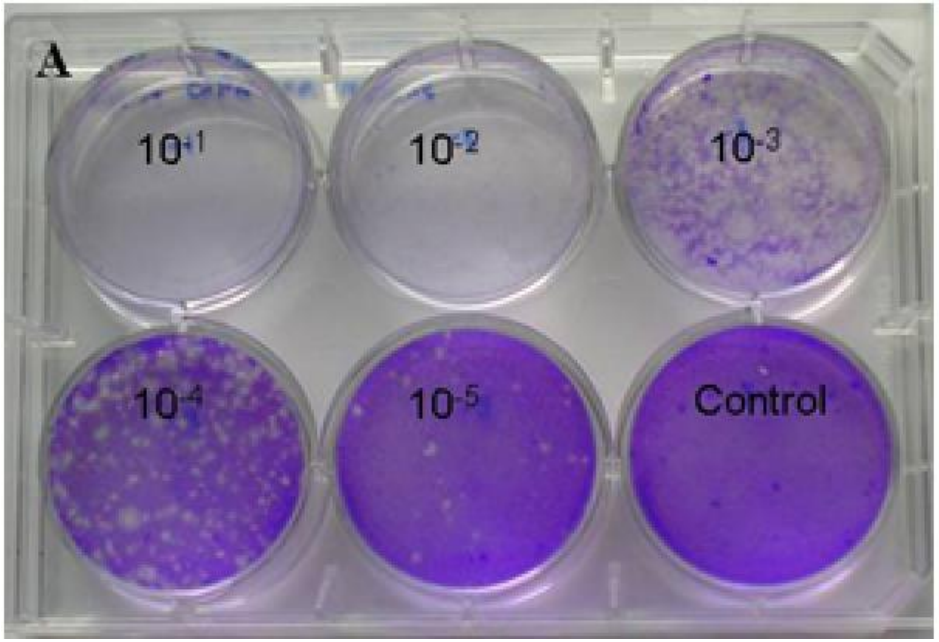
- Infectious Titer
  - Essentially, a measure of how well a virus is able to infect target cells, given as a measure of concentration
  - X infections/Y amount of virus provided

Infectious Titer Methodologies

- Viral Plaque Assay – Time-tested Gold Standard of Virology
  - Cheap, effective, time-consuming, user-dependent accuracy
- Quantitative Polymerase Chain Reaction (qPCR)
  - High accuracy, high, cost, requires Virus specific primers

Current Collaborators In Eastern US

- Viral Plaque Assay
  - Serially dilute viral preparations, infect plates of confluent cells
    - Incubation takes place until a cytopathic effect is observed. Due to the slow moving nature of Lentivirus this process was taking over **two weeks get a reliable number of cell morphology changes/deaths!!!**
    - Apply agar gel stop slow diffusion of virus
    - Count the number of cytopathic incidents per each serial dilution
    - Average is you Infectious Titer
  - Pretty simple but there are some drawbacks
    - User to user variability
    - Not all viruses cause drastic disruptions to cell morphology or death
    - **TIME**



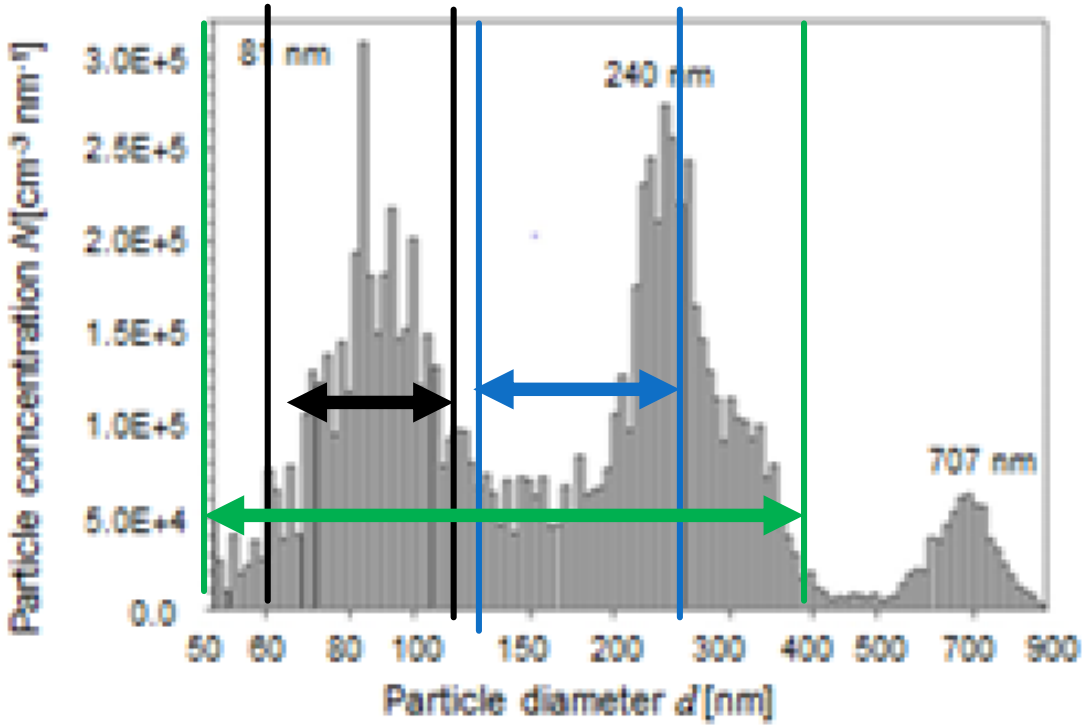
http://theses.ulaval.ca/archimede/fichiers/24866/ch05.html

Goals with ViewSizer

- Find a size range that gives a comparable, reproducible number that correlates to the data produced from IT assays
- Measured whole spectrum of sizes in each sample, but counted based upon three windows
  - one virus
  - dimer
  - All-detritus to aggregates

What we learned from their Data

- Sadly, NDA prevents usage of real data
- Broken into three ranges



Data Collection and Anaylsis

- Averaged data across multiple replicates of each retain sample across multiple users, including myself and employees of Company during their one month rental
- Compiled everything to get an average ± STD for each sample run
- Plotted sample counts for each size range versus known infectious titer values
- Results indicated that measurement results from a wide range of sizes was the best predictor of IT value
  - Best R^2-value of 0.9 (vs 0.7 and 0.6 for smaller ranges)
- Turns out the count from ViewSizer at 0-400 nm and IT values were typically only an order of magnitude apart for this virus

Take-Aways From Infectious Titer Measurements

- Customers already owned competitive instruments with a single laser, application had not panned out
- With some application work, we were able to find a consistent and robust correlation to Infectious Titer values
- Cutting their timeframe from several weeks to around 20 minutes/sample
- Significantly Increasing reliability/reproducibility
- Crucial to measure ENTIRE range of sample to correlate it IT
- Matches literature for similar viral vectors: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4139191/#bib9
- Virology. 2014 Aug; 462-463(100): 199–206. doi: 10.1016/j.virol.2014.06.005

Take-Aways From Infectious Titer Measurements

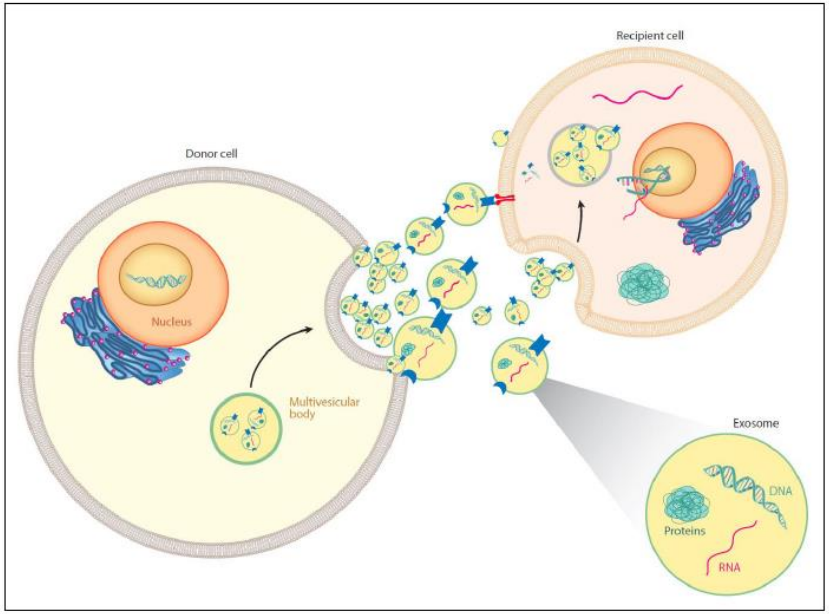
- Advantages of NTA over Comparative Techniques
  - qPCR is costly and requires an extensive prior knowledge of the viral genome sequence.
  - Primers have to be derived against specific non-mutating regions of the viral genome and standards need to be derived from plaque assays for each virus species tested.
  - However, as qPCR detects the presence of small, specific regions of nucleic acid, it cannot quantify invasive viral particles.
  - **No need to lyse cells, counting whole particles and fractions in solution**
    - qPCR uses isolated nucleic acid lysed from cells or precipitated from solution
    - Accuracy of the readout depends on the purity of the sample.
  - Quickly and accurately determines viral titers and sample purity will be extremely important for analyzing preparations of viruses in the expanding viral therapy market.
  - Easily reproducible across multiple end users
  - Only sample preparation is serial dilution from original aliquot

Additional applications of interest for Viral Production

- In addition to addressing IT customers have found Viewsizer to be an excellent tool for:
  - Stability testing via freeze/thaw cycles
  - Watch aggregates form over time as freeze thaw cycles pass in -80 freezer
  - Enables look at stability of product, obtain information for product volatility
- Excellent tool for examining batch to batch variability via sizing and concentration across polydisperse ranges
  - Used as a QC tool to look for aggregates in post-production
  - Enables end-users to look at variations in production that could have caused upsets in process

Extracellular Vesicles: Exosomes

Extracellular Vesicles (EVs)

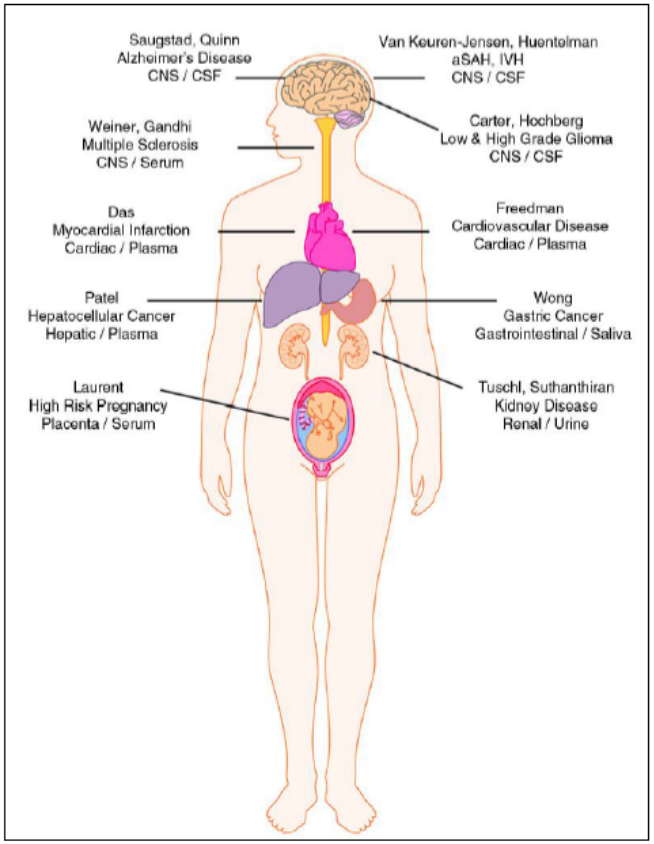


Kourembanas (2015), ***Annu. Rev. Physiol.***

EVs are released by many cell types, in both physiological and pathological states.

EV Locations

EVs are found in Biofluids



Quinn et al. (2015), ***J Extracellular Ves***

Implications:

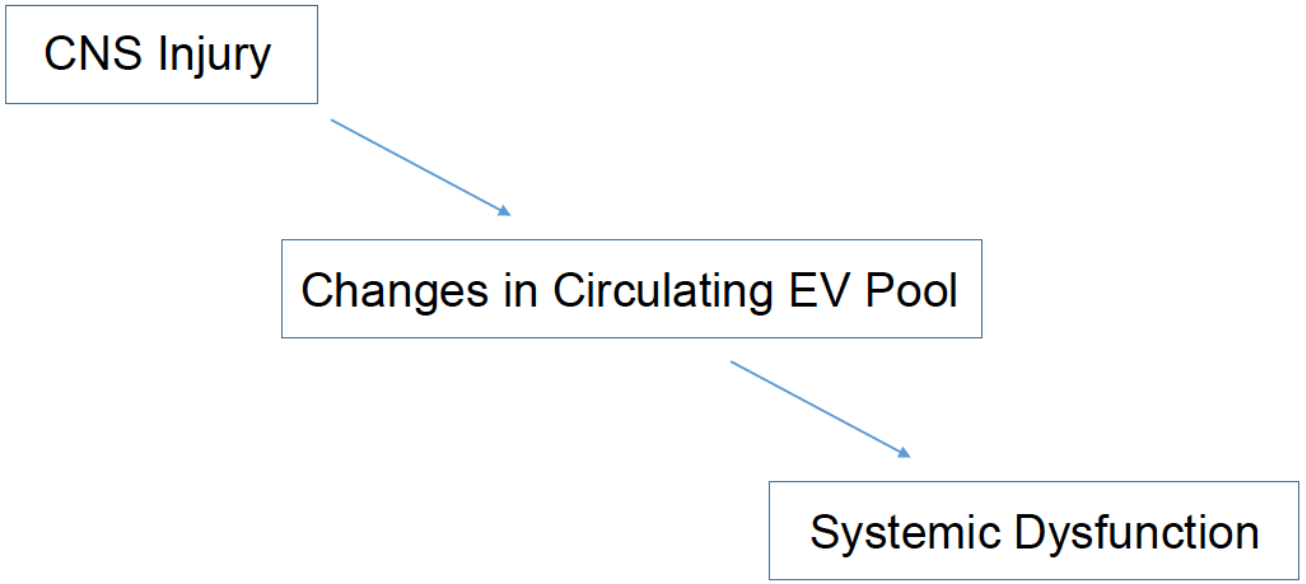
(1) Potential for long-range communication
- Brain-gut
- Brain-peripheral immune system

(2) Novel Biomarkers

(3) EV Therapeutic Engineering

EV as biomarkers for injuries

Working Model



Are there any changes in the number and size distribution profile of plasma extracellular vesicles after spinal cord injury?

Experimental Design

Nanoparticle Tracking Analysis (NTA)

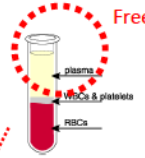
Spinal Cord Injury Contusion OR No injury

Differential Centrifugation

Collect Blood

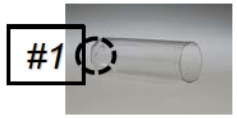
500g, 15 min RT

2500g, 10 min, RT (2x)



Platelet Free Plasma

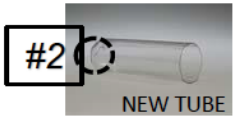
Large MVs



12,000*g*, 1 hr, 4°C
MLS-50
Swinging Bucket Rotor

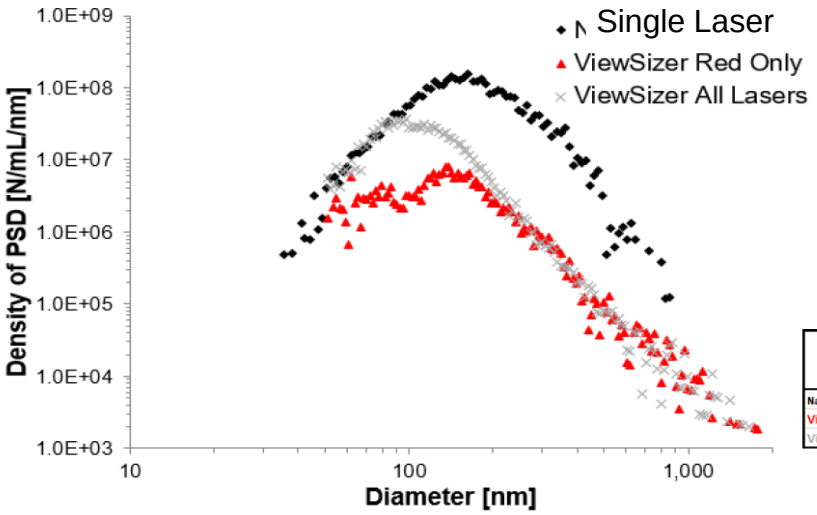
Transfer Supernatant

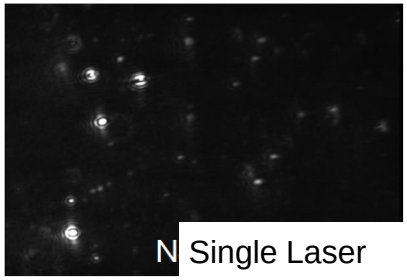
EXOs and small MVs

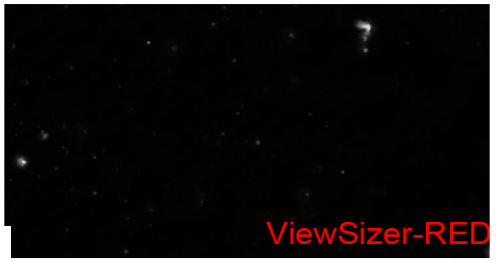


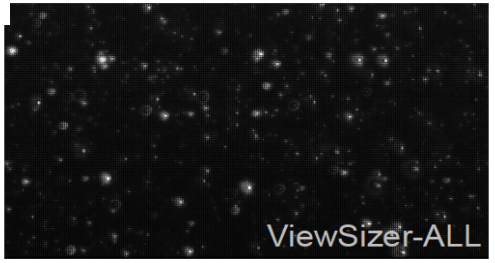
110,000*g*, 2 hr, 4°C
MLS-50
Swinging Bucket Rotor

Red wavelength





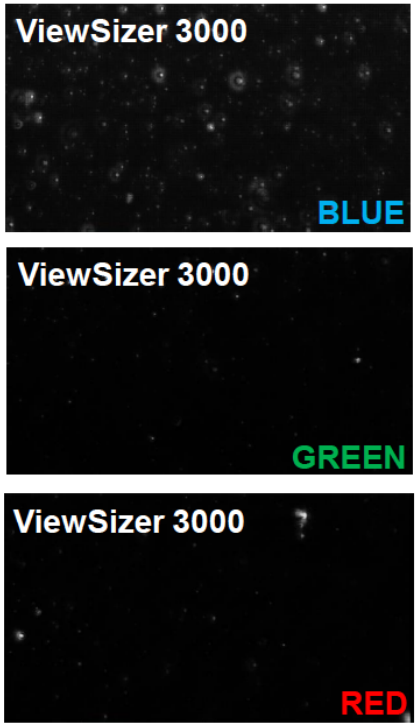


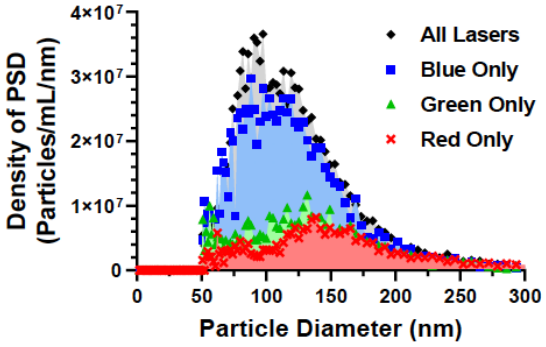


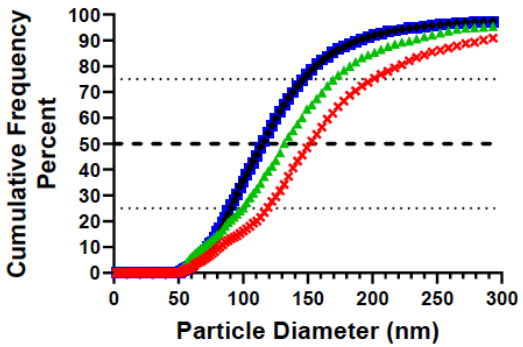
	Average Size (nm)	Median Size (nm)	Particle Concentration (part./mL)
NanoSight LM10	204	186	2.20E+10
ViewSizer Red	178	153	8.65E+08
ViewSizer All	131	116	3.03E+09

- Compares a single wavelength of the ViewSizer (red) to a similar instrument and against all three ViewSizer wavelengths
- Results indicate significant shift in data to the left (smaller particle size) using using all three lasers
  - Significant portion of the sample is picked up only using all three wavelengths
  - Demonstrates utility of multi-laser NTA and how interrogating a broader particle size distribution leads to a better characterization and understanding of a polydisperse sample

Influence of Laser Wavelength on Particle Detection



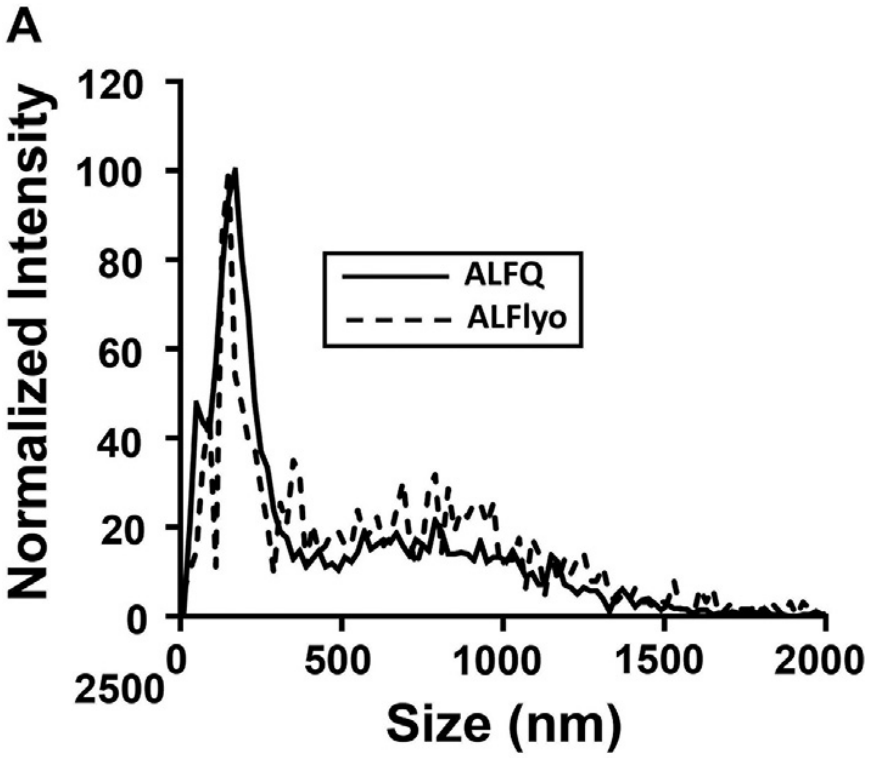




	Average Size (nm)	Median Size (nm)	Particle Concentration (part./mL)
All Lasers	131	116	3.03E+09
Blue Only	132	116	2.58E+09
Green Only	153	132	1.07E+09
Red Only	178	153	8.65E+08

EVs isolated from plasma require higher energy wavelengths for accurate analysis

Liposomal Adjuvants



- Work at Walter Reed Army Institute of Advances Research (WRAIR)
- Singh et al., Biochemical and Biophysical Research Communications 529 (2020) 362e365

Conclusions

- NTA is a powerful tool for examining polydisperse samples, difficult to impossible to properly characterize with Size and Concentration via other means!
- For biologics looking at a size range from ~50nm - 2000nm (2 micron)
  - Expanded via fluorescence, can also be used to interrogate subsections of complex biological mixtures
- Currently have made significant progress in
    - Viruses/Gene Therapy
    - Extracellular Vesicles
    - Exosomes
    - Adjuvants
- Thank you all!!

Citations

- https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4139191/#bib9
- Virology. 2014 Aug; 462-463(100): 199–206.
  doi: 10.1016/j.virol.2014.06.005
- https://www.mirusbio.com/applications/high-titer-virus-production/lentivirus-production
- https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3044498/
- http://theses.ulaval.ca/archimede/fichiers/24866/ch05.html
- https://en.wikipedia.org/wiki/Exosome_(vesicle)
- https://en.wikipedia.org/wiki/Antibody#/media/File:Antibody.svg

QUESTIONS?!

Thank you all for your time!

- Thank you:
  - Niaz Khan, MD/PhD
  - Jeff Bodycomb, PhD
  - Bill Travers