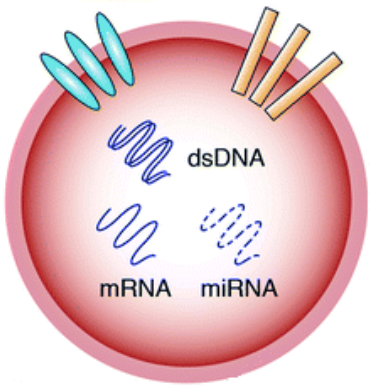
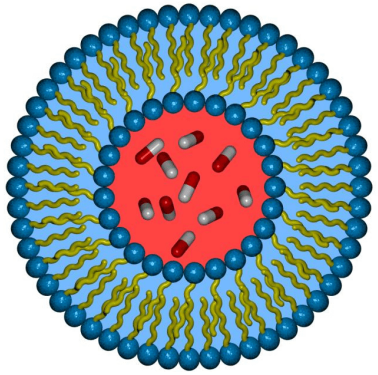
Improved Methods For Fluorescent Labelling And Detection with Nanoparticle Tracking Analysis (NTA)

Aymeric AUDFRAY, PhD
Field Application Scientist, Pharma & Food, France
aymeric.audfray@malvern.com

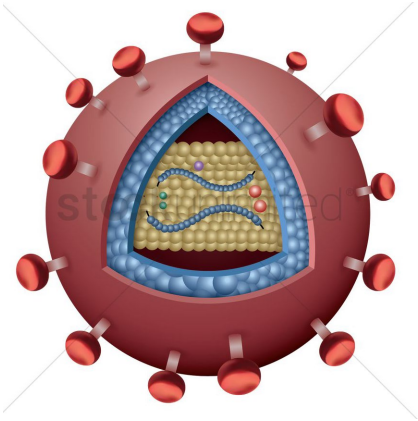
Exosomes



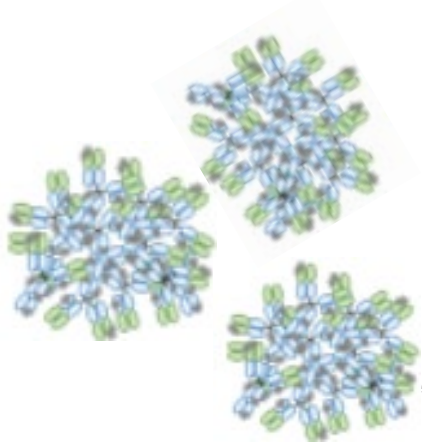
LNPs (Nanoparticles)



Viruses

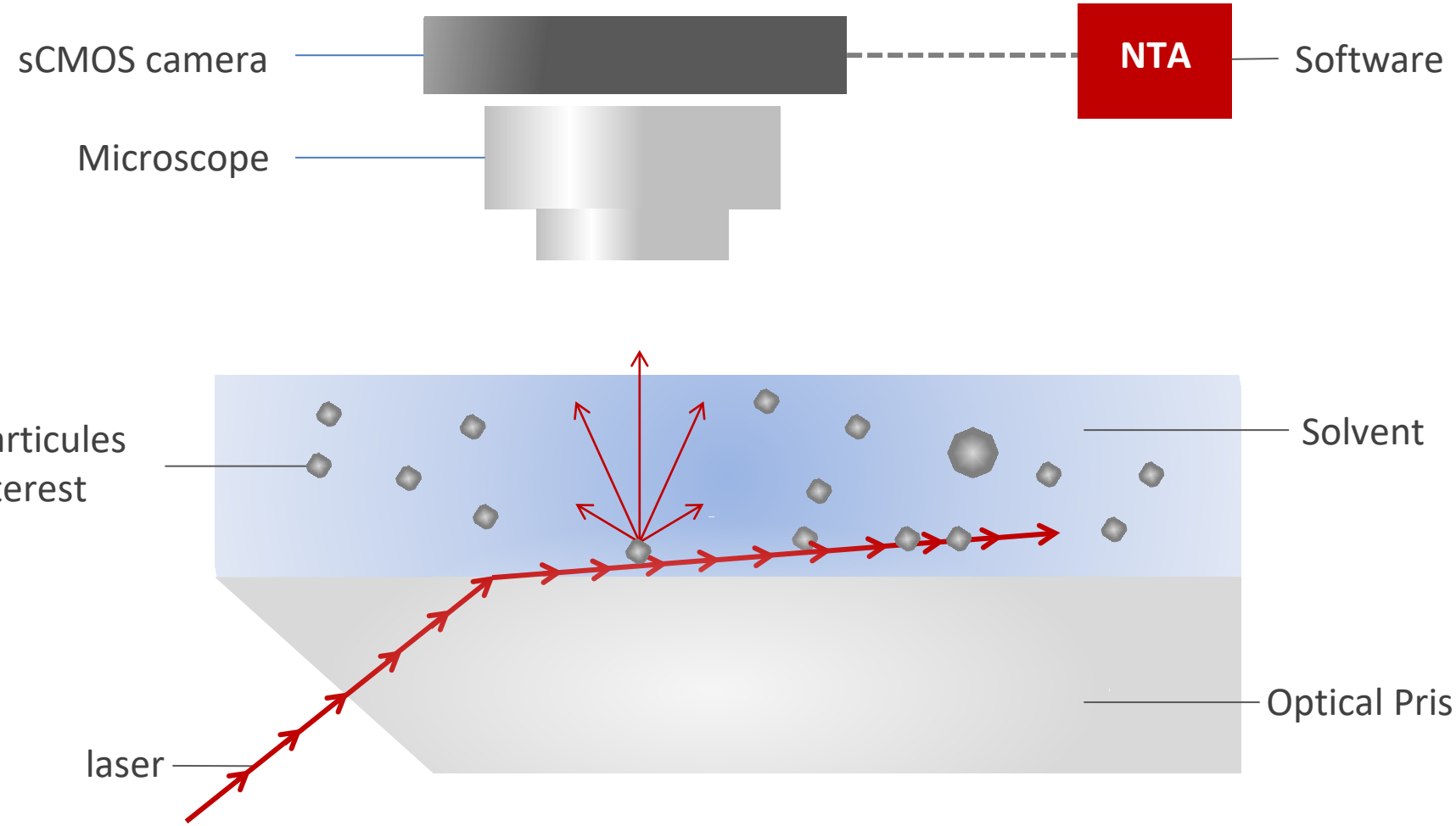


Protein Aggregates



Nanobubbles

Principle of Nanoparticle Tracking Analysis



Patent:

Bob Carr

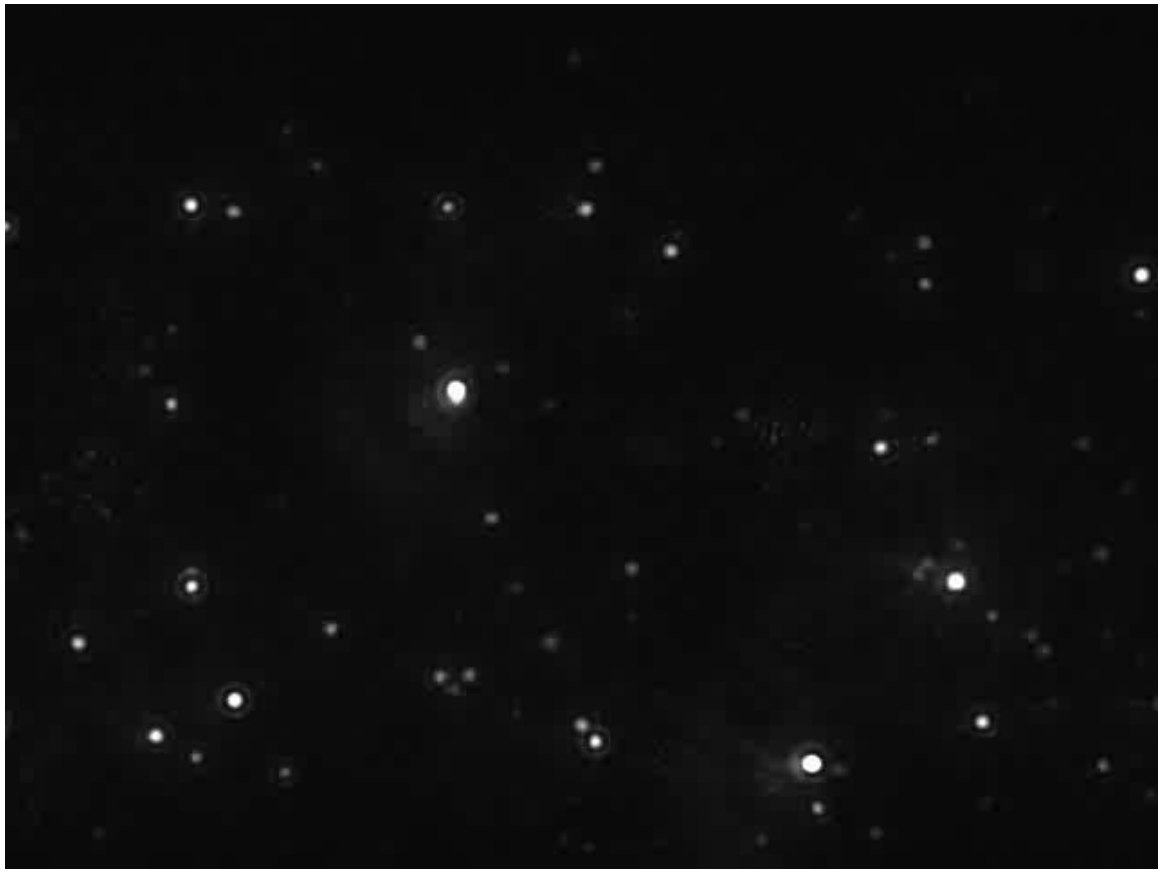
(CEO Nanosight 2003)

PCT/GB03/001827

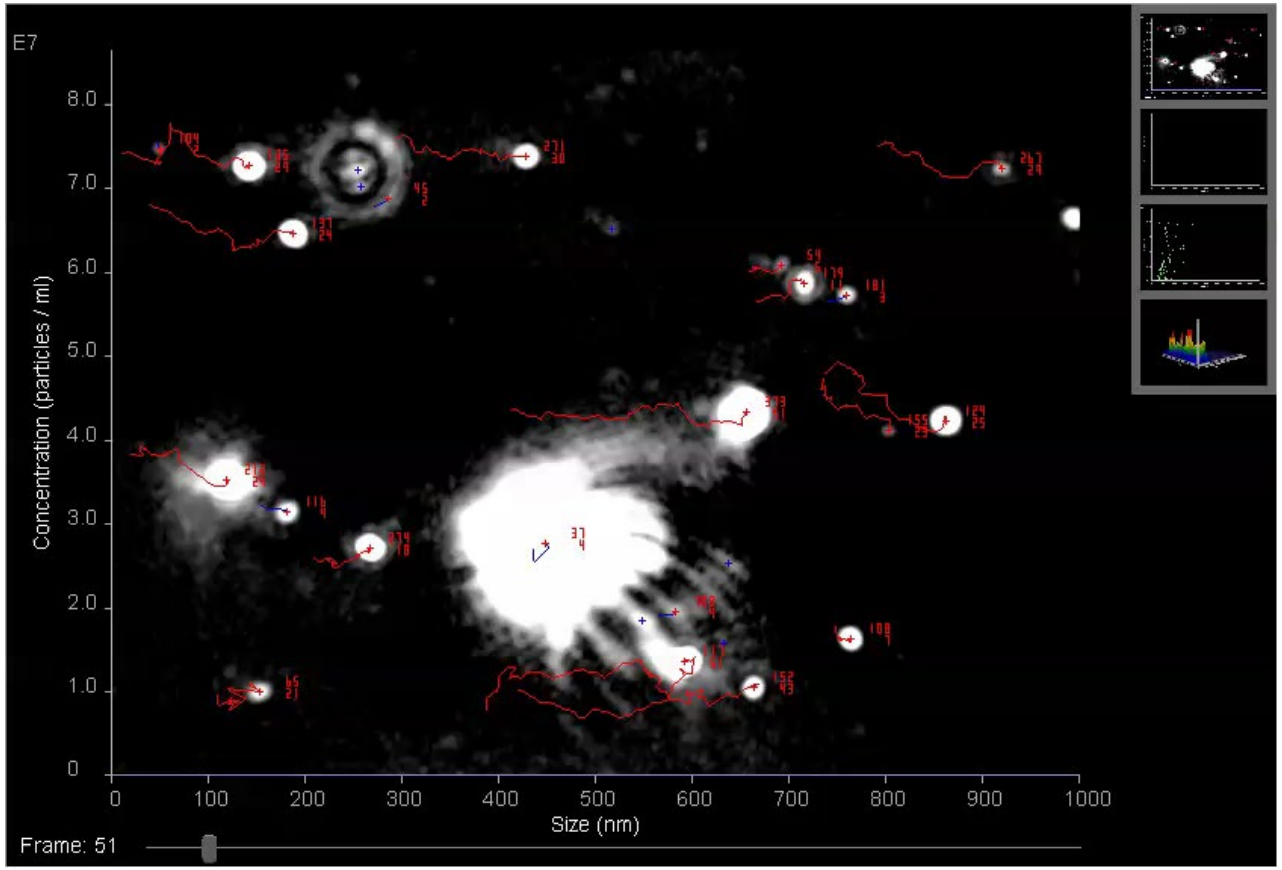
Principle of Nanoparticle Tracking Analysis

Malvern Panalytical
a spectris company

Video-captures = raw data



Processing = Tracking of Brownian Motion



Principle of Nanoparticle Tracking Analysis

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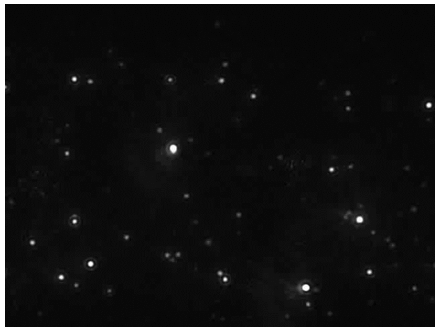
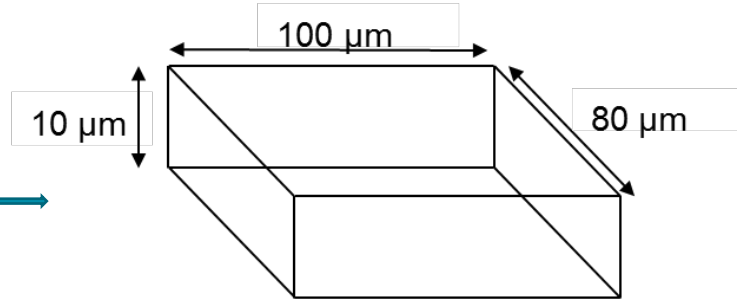
Average number of particles / frame

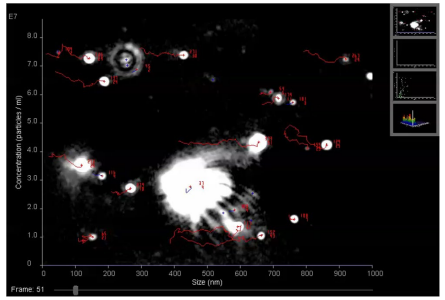




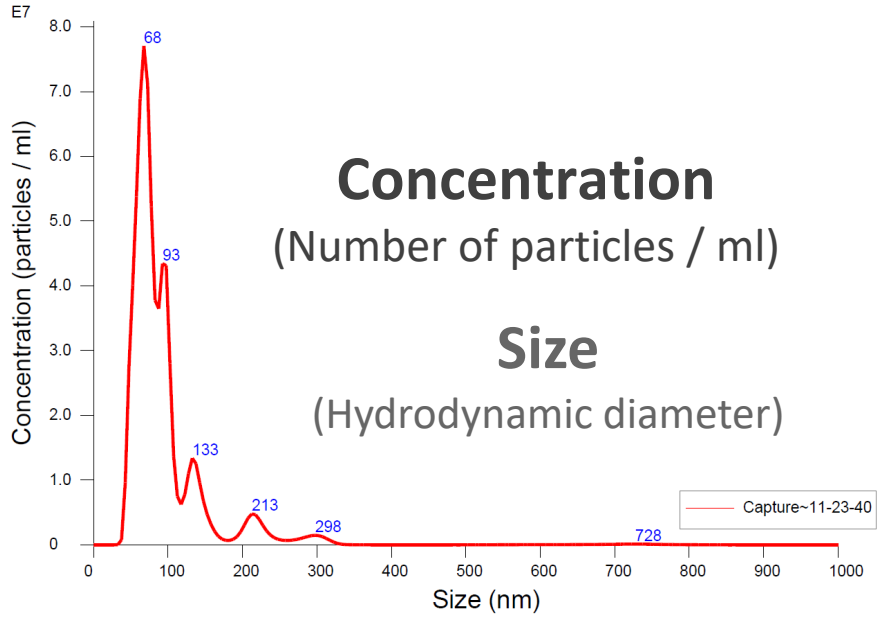
Known Volume

Tracking of Brownian Motion

= Diffusion Coefficient (m^2/s)



$$D_t = \frac{K_B T}{3\pi\eta d_h}$$



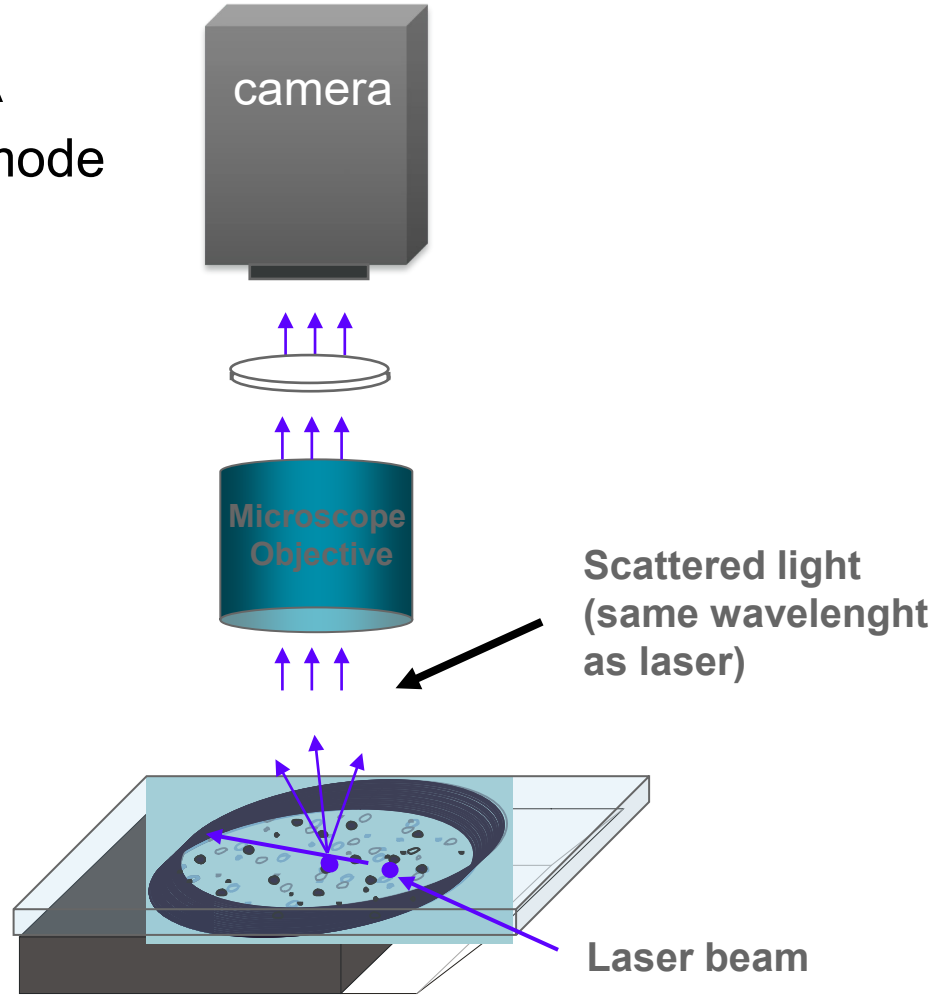
Concentration
(Number of particles / ml)

Size
(Hydrodynamic diameter)

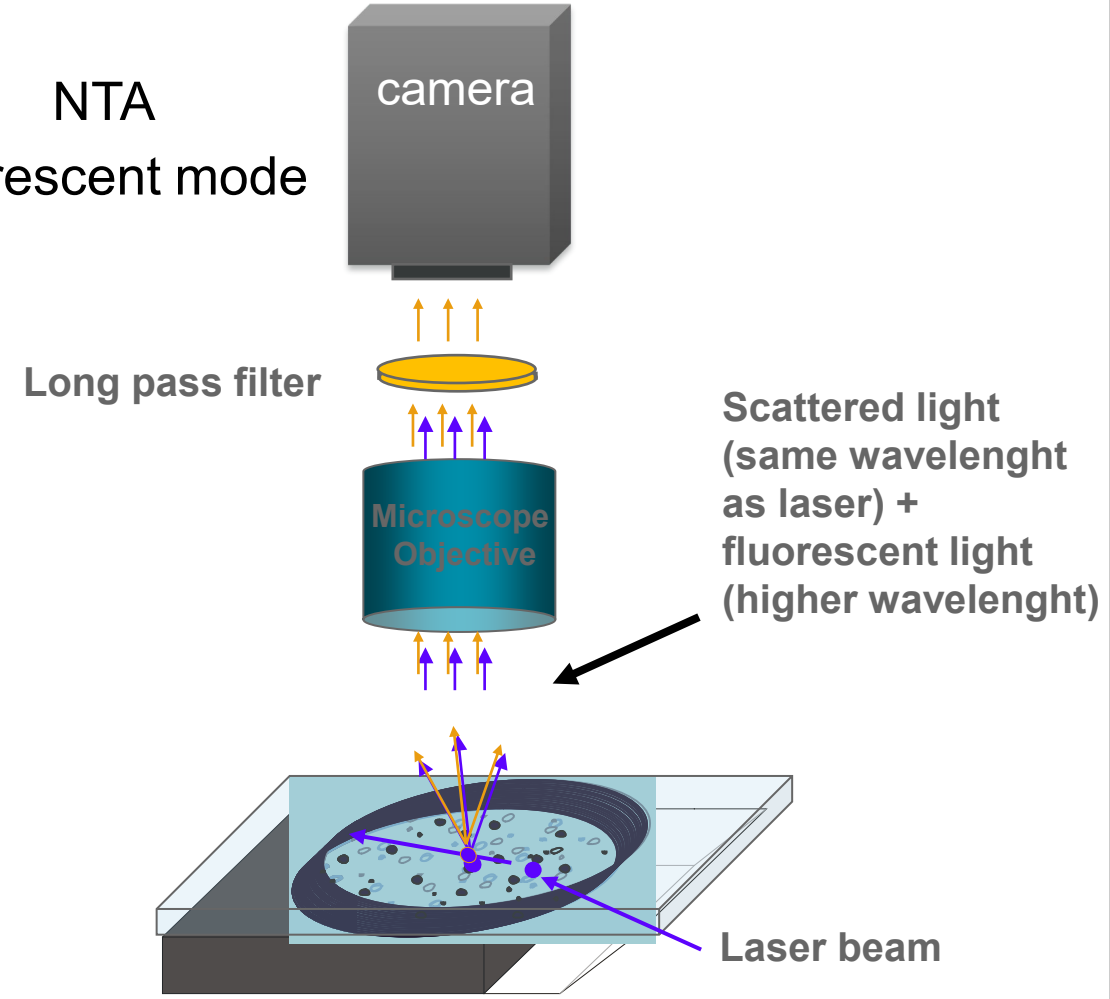
NTA vs Fluo-NTA

Principle

NTA
scatter mode



NTA
fluorescent mode



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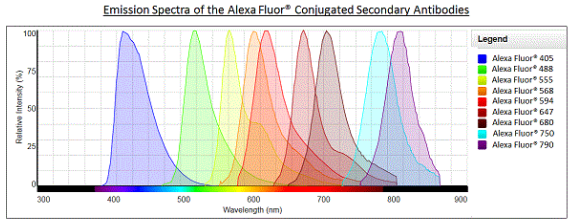
General Tips for Fluorescent Measurements

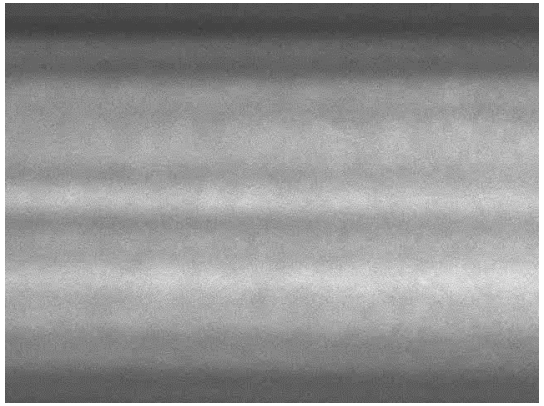
- Fluo-NTA is always more difficult than scatter mode
  - Samples are more complex (labelling) and settings are more complicated (signal)



- Minimum number of fluorescent dyes by particle:
  - 30-50 for small molecule dyes
  - 3-5 for Quantum Dots
  - Dye excitation maxima must match laser wavelenght (405nm, 488nm, 532nm, 642nm)

- Do all operations likely to reduce photobleaching:
  - Trigger cable (present in all instruments now)
  - Use syringe pump
  - Connect inlet tubing to the right port of the LVFC
  - Protect syringe, tubings,… from light.

- Minimize unbound fluorescent dye (generate background noise)



- Check focus setting (different focal plan between scatter and fluorescence)
- Be careful at the dilution (depends on the % of labelling)
- Use advanced camera settings

Advanced Camera Settings

Fluo-NTA

- Usually CL is at 16 (maximum) for fluorescent NTA

- Possibility to increase the signal with advanced camera settings
  - Push Camera Shutter/Camera Gain to the right
  - Adjust left (contrast) and right (light) treshold of Camera Histogram

Labelling Strategies

Fluo-NTA

- EVs labelling is the most common application for fluo-NTA
- Membrane Labelling:
  - Exoglow (488nm laser, https://systembio.com/shop/exoglow-nta-fluorescent-labelling-kit/)
  - CellMaskOrange (532nm laser)
  - Specific to EVs over protein aggregates, easy labelling with hundreds of dyes by particle
  - Non specific to surface markers
- Antibody labelling
  - Highly specific to surface biomarkers
  - Can be challenging to obtain sufficient signal (30-50 tags per particle)

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Fluorescent Labelling of membranes and analysis by Malvern Panalytical Fluo-NTA

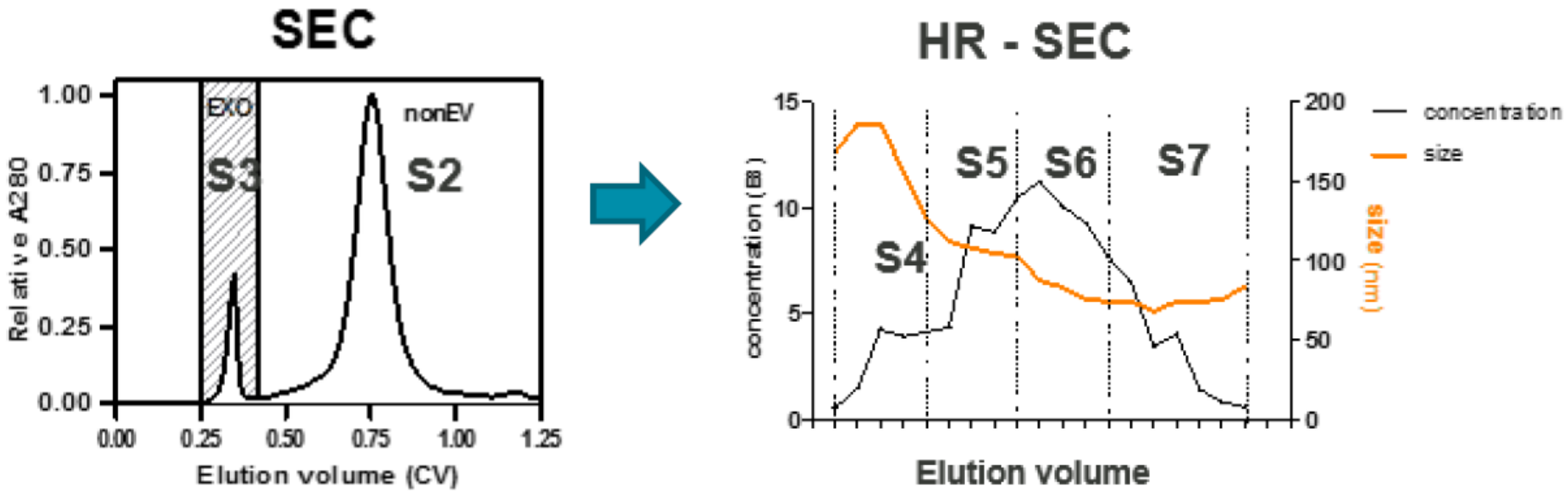
Aymeric AUDFRAY, PhD
Field Application Scientist, Pharma & Food, France
aymeric.audfray@malvern.com

NanoSight Characterisation of Fractionated Exosomes (EVs) Labelled with CellMask™Orange

Agnieszka Siupa[1], Eduard Willms[2], Pauline Carnell[1], Imre Mager[2]
[1]*Malvern Panalytical, GrovewoodRoad, WR14 1XZ, Malvern, Worcestershire, UK*
[2]*Department of Physiology, Anatomy and Genetics, Le Gros Clark Building, South Parks Road, Oxford*

Purification of EVs

- EVs were isolated from cell culture supernatant of SKOV3 cells
- Differential centrifugation approach
- Size exclusion chromatography **(SEC)** /High resolution size exclusion chromatography **(HR-SEC)**



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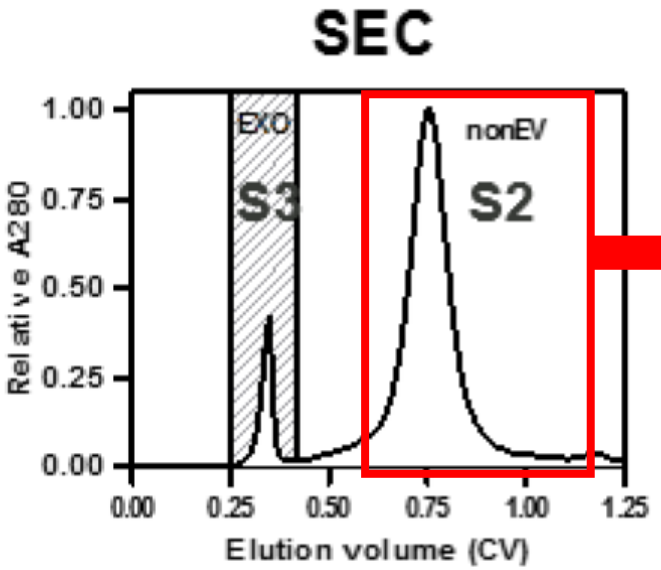
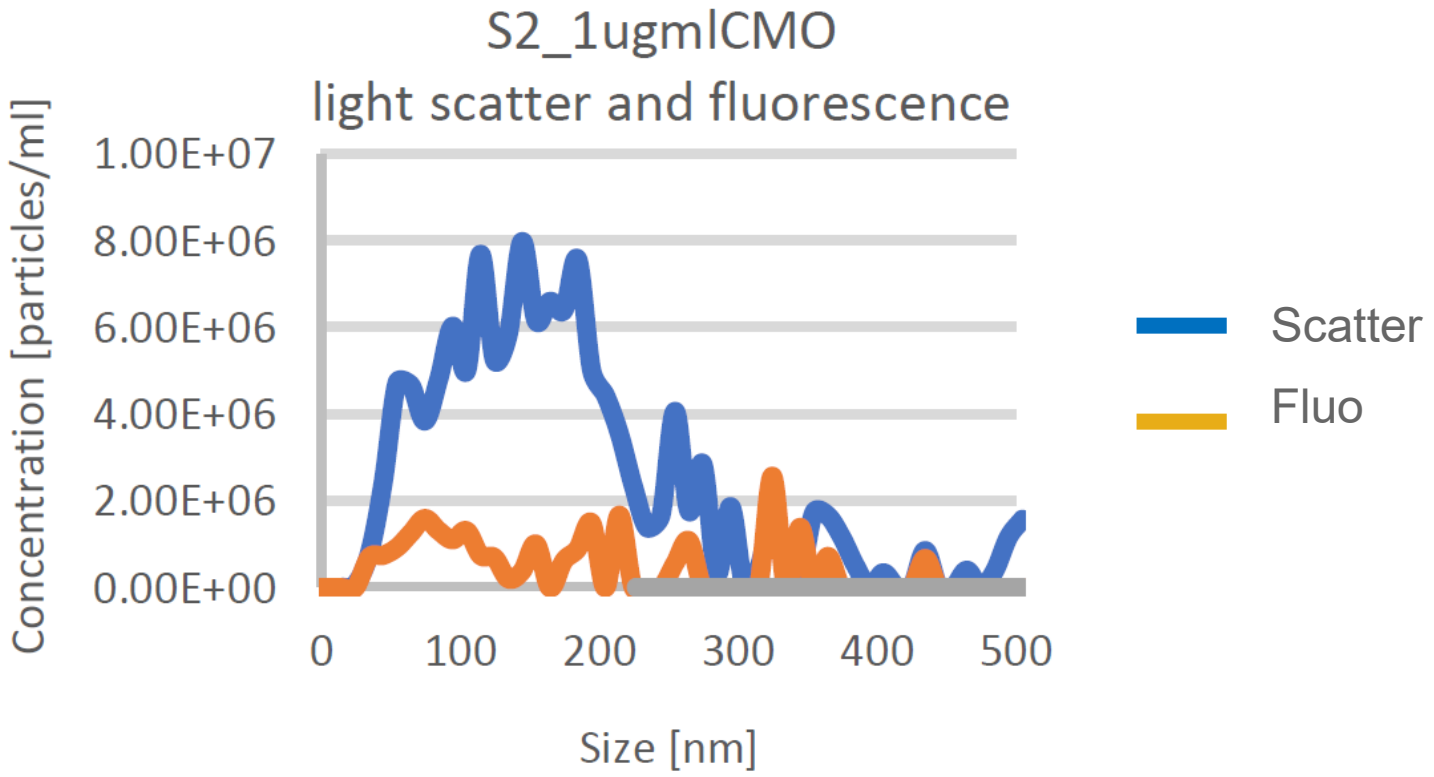
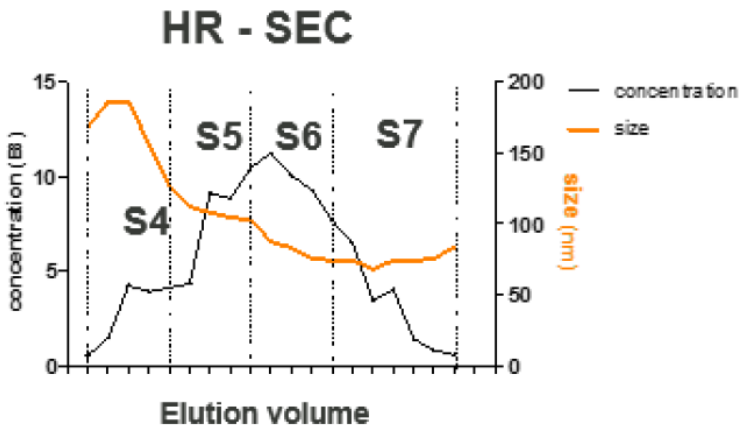
Labelling of EVs with CellMaskOrange® (CMO)

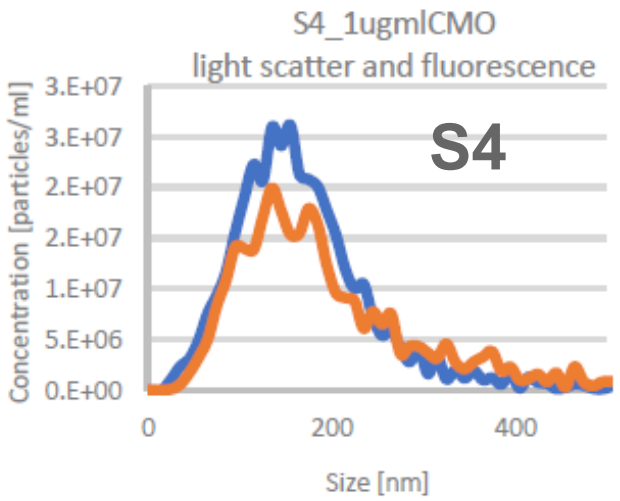


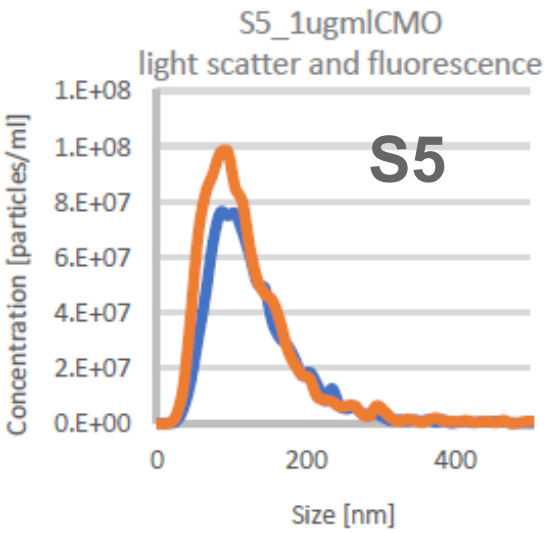


- Fraction S2 contains Nanoparticles that absorbs UV (most likely protein agregates)
- **No fluorescence signal**
- **No labelling by CMO**

Comparison of scatter and fluo results

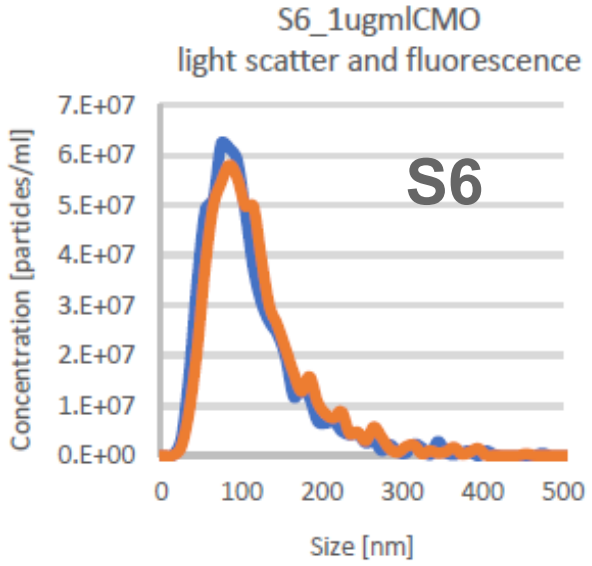


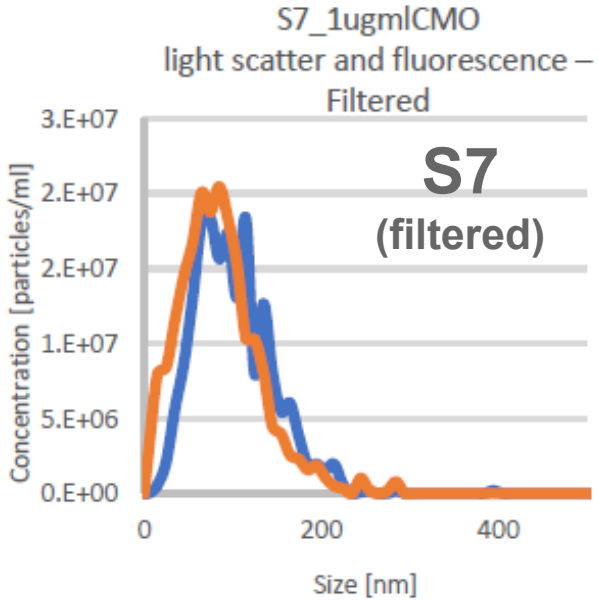




Scatter

Fluo





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Conclusion

- Efficient and simple method to analyse **only** EVs in a samples using Fluo-NTA
- Universal Protocol developped by Malvern Panalytical – **Available on demand**
- Standardization of EVs concentration and size analysis
- Other protocol are being developped with different dyes and wavelenghts

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Improved methods for fluorescent labeling and detection of single extracellular vesicles using nanoparticle tracking analysis

Received: 24 January 2018
Accepted: 26 July 2019
Published online: 23 August 2019

Kristen E. Thane, Airiel M. Davis & Andrew M. Hoffman

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nature research

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- EVs extracted from:
  - Canine Mesenchymal Stromal cells (MSCs) of placental origin
  - Human Embryonic Kidney cells ATCC CRL-1573 (HEK-293)
- Isolation using Ultracentrifugation
- Stored at 4°C before immunolabelling (0-4 days)
- NTA Measurement:
  - NS300 with syringe pump
  - Laser 488nm + long-pass filter 500nm
  - SOP: 5x30-60s with flow mode (minimum of 1000 valid tracks)

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- Labelling protocol:
  - Direct:
    - anti-CD9-Qd655
    - anti-CD81-APC
  - Indirect:
    - anti-CD9 + Secondary Ab-Qd655
    - **anti-CD9-biotin + Streptavidin-Qd655**
    - anti-CD81 + Secondary Ab-Qd655
    - anti-CD81-biotin + Streptavidin-Qd655

  - 10^{10} EVs + 1µg of Anti-CD in 100µl of PBS, 4°C Overnight,
  - Ultracentrifugation 100 000 g, 2 hours and resuspension in 50-100µl
  - Anayzed immediatly (after dilution)

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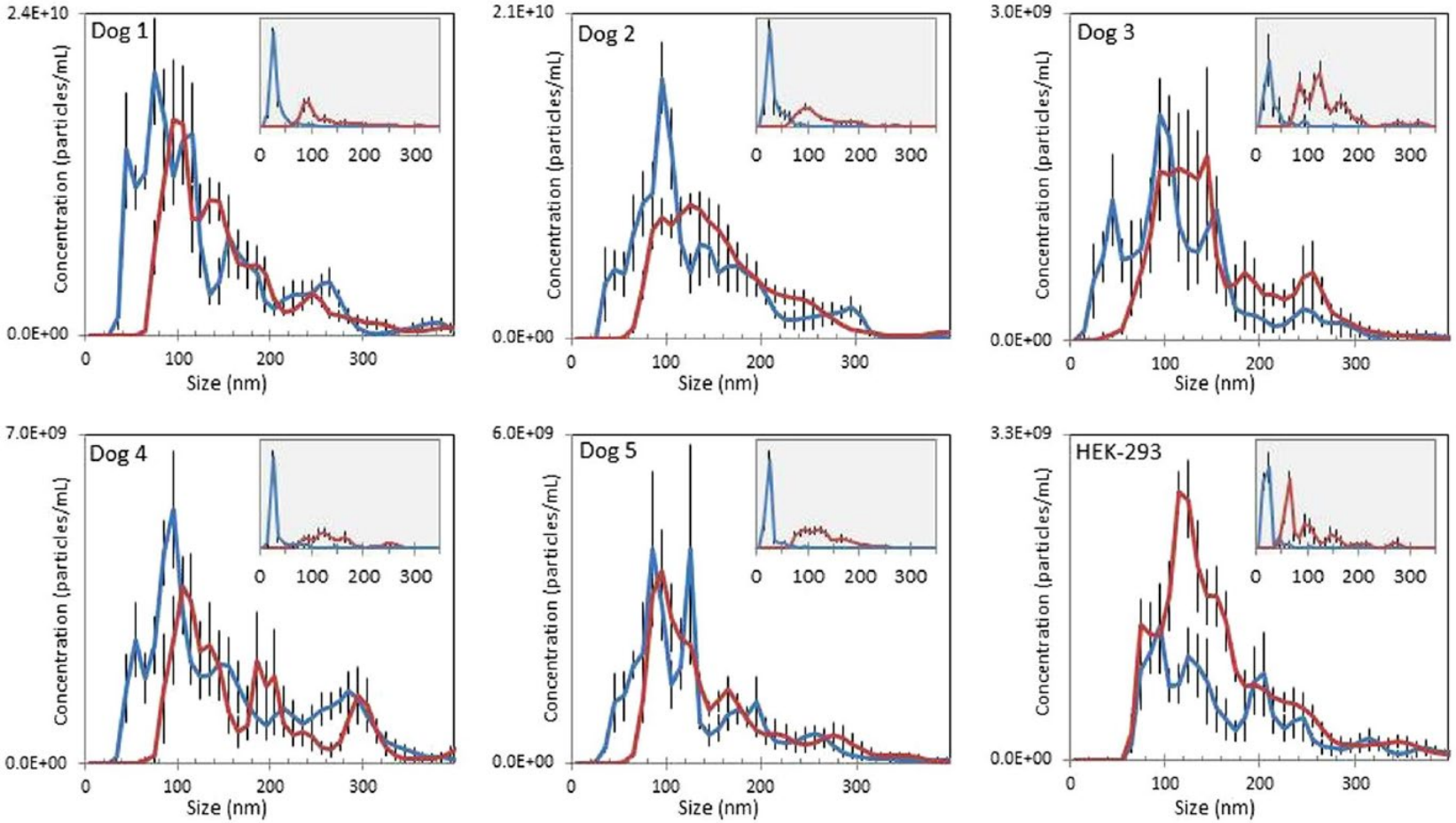
- anti-CD9-biotin + Streptavidin-Qd655 :



Scatter Mode

Fluo Mode

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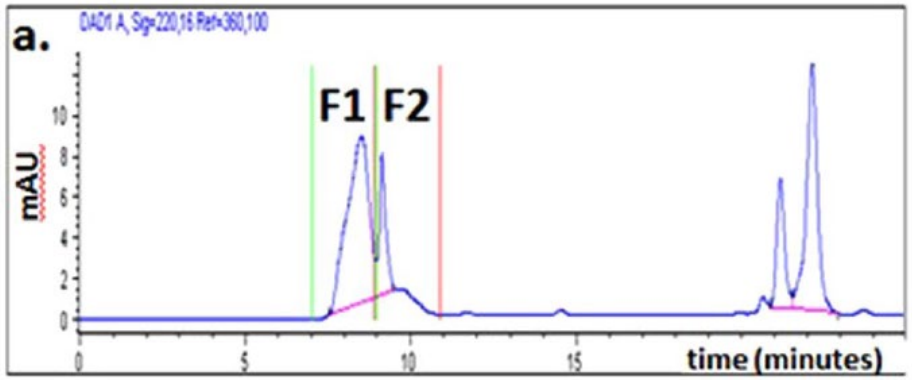
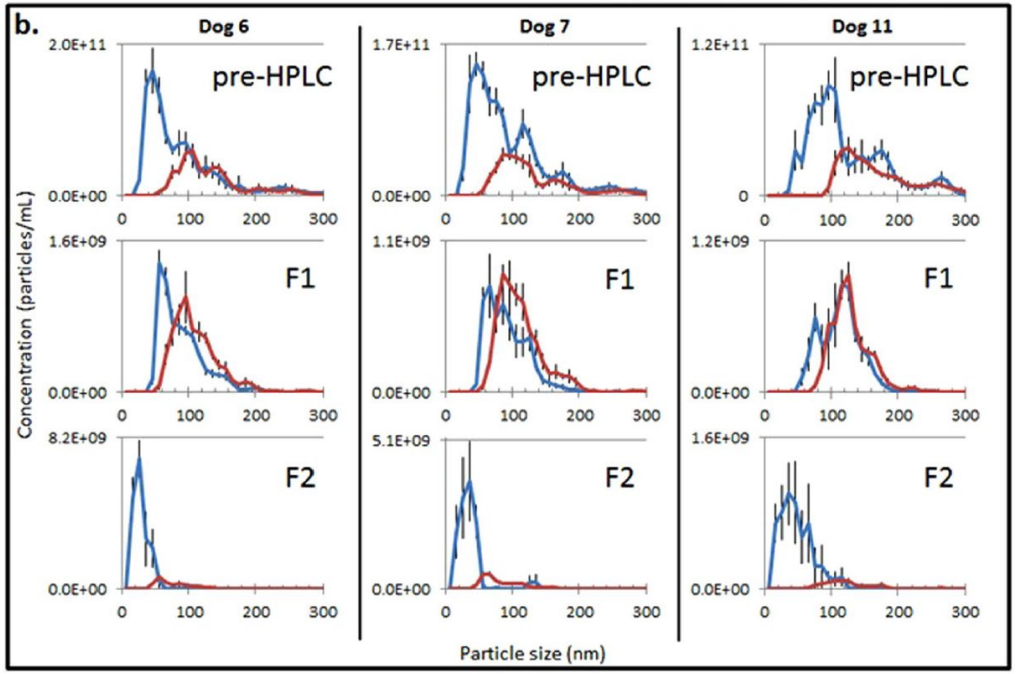
- Purification of free Streptavidin-Qd655
- 2 methods:
  - Capture of Strep-Qdot using Biotinylated-beads (not shown here)
  - HPLC



Fluo Mode Scatter Mode



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- Conclusion:
  - Development of a method to standardize EVs characterization using Fluo-NTA (protocol and analysis)
  - Use of Quantum dots with 488nm laser
  - Best approach: CD9-biot + Streptavidin-Qd655
  - Develop a method to remove free fluorophore using HPLC or Biotinylated-beads

General Conclusion

- Scatter mode NTA is already used as routine for NPs characterization
- Fluo-NTA can be more challenging (depending on samples)
- Different labelling approaches (membranes, markers,…)
  - CellMaskOrange labelling is universal and available on demand
  - Protocols from bibliography
  - Other specific protocol have to be developped
- Importance of labelling protocol to have sufficient signal/decrease photobleaching
  - One good example: indirect labelling using **Ab-biot + Streptavidin-Qdots**
  - Use of **Qdots** seems very promising
- Support from Malvern Panalytical
- Support from « Concept Life Science » for method development

Concept Life Sciences
a Malvern Panalytical brand

Malvern Panalytical
a spectris company

THANKS FOR YOUR ATTENTION

Labelling of EVs with CellMaskOrange® (CMO)

- CellMaskOrange® is a dye able to bind specifically to lipid bilayers
- 10µl of 1µg/ml CMO + 10µl EVs (non diluted)
- Incubation 10min at room temperature
- Dilution of samples to optimal concentration range for NTA measurements
- Analyzed on NS300 device
  - 532 nm laser
  - 565 nm fluorescence filter
  - Flow mode
  - Concentration upgrade
- Analysis have been made using both scatter and fluorescence mode