ESTIMATION OF PROTEIN AGGREGATE DENSITY USING SEDIMENTATION COMBINED WITH MICRO-PARTICLE TRACKING

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NIST
National Institute of Standards and Technology
U.S. Department of Commerce

MATERIAL
MEASUREMENT
LABORATORY

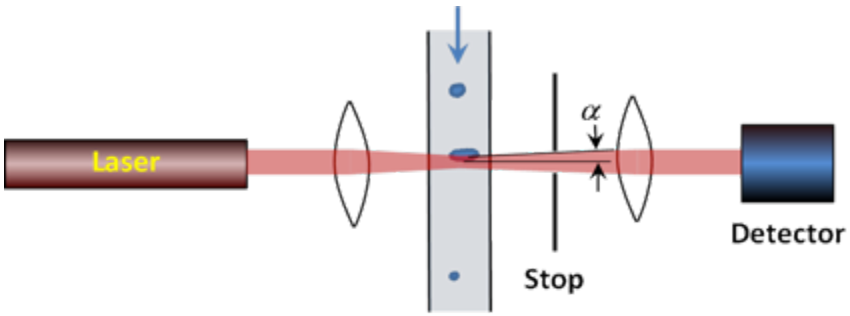
NIST

Outline

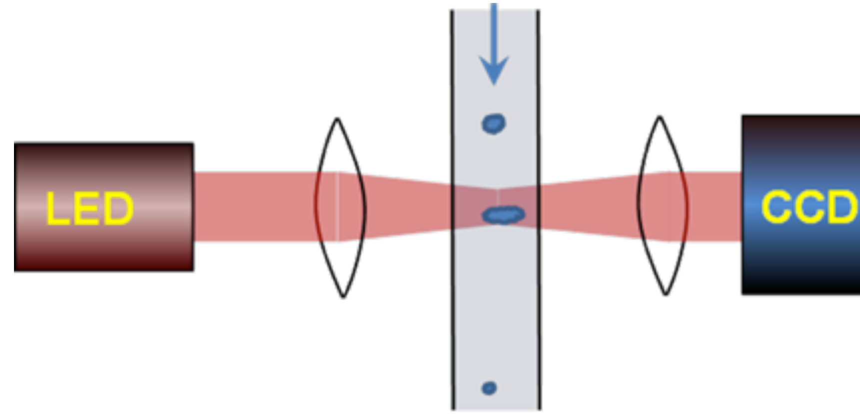
1. Possible improvements to Flow Microscopy Analysis
   a. Shape effect
   b. New image processing algorithm
2. Aggregate Density measurements based on sedimentation
3. New Reference Materials
   a. Fluoropolymer aggregate simulants
   b. Other materials under development

Subvisible Particle Sizing Methods

Light obscuration (LO) ΔI ~ area

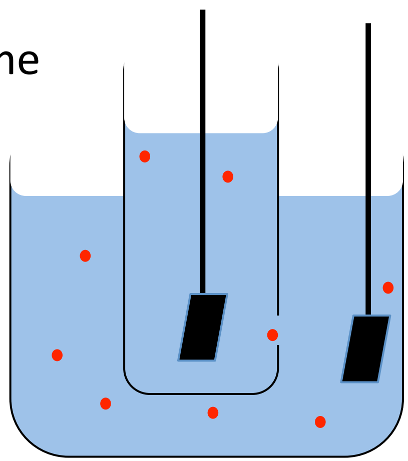


Flow Imaging (FI) area

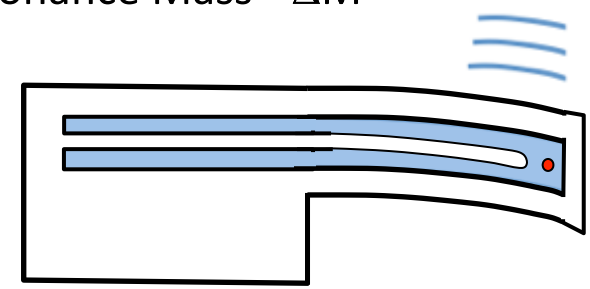


Electrical Sensing Zone (Coulter) (ESZ)

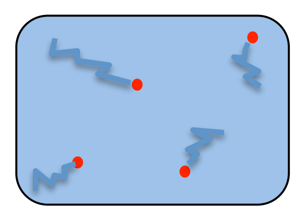
ΔR ~ volume



Resonance Mass ΔM



Particle tracking D (related to $d_{hydrodynamic}$)

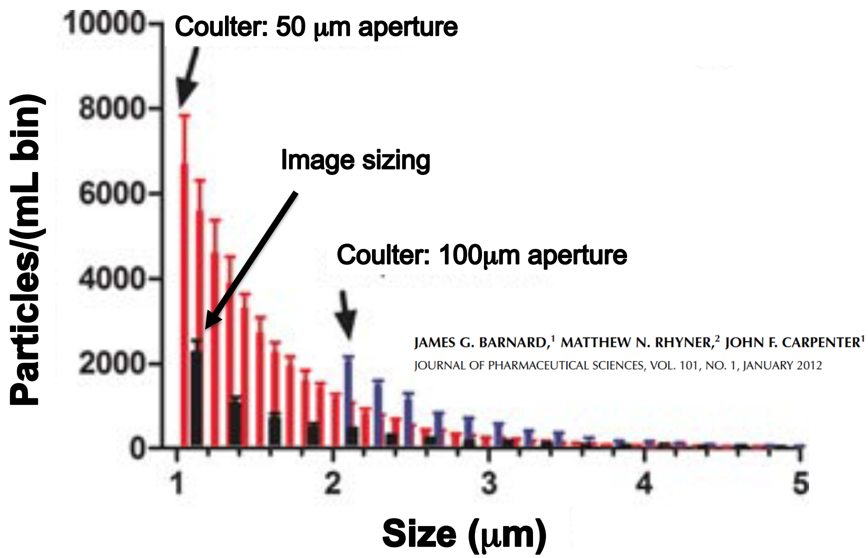


Measured diameter, concentration for polystyrene beads.

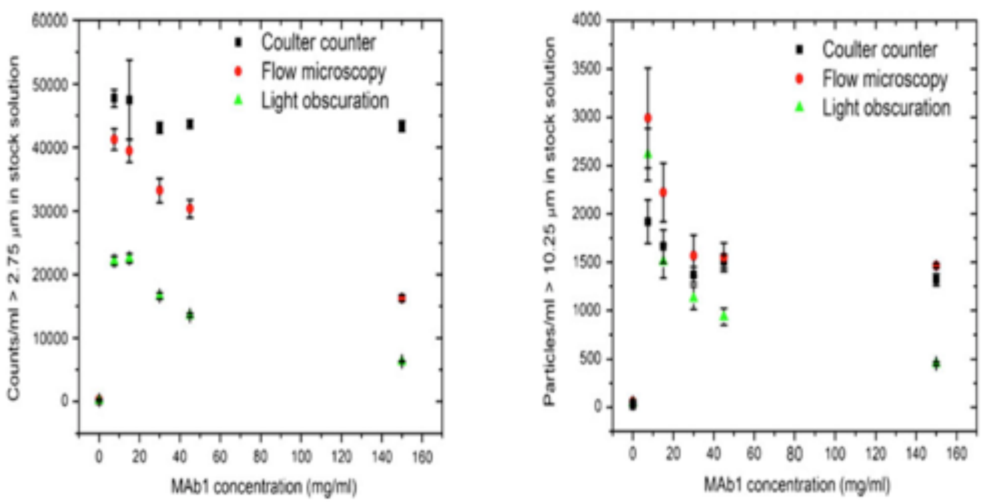
Nominal dimension	ESZ (Multisizer)		FI Instrument A		FI Instrument B		LO	
d (µm)	d (µm)	N (ml^{-1})	d (µm)	N (ml^{-1})	d (µm)	N (ml^{-1})	d (µm)	N (ml^{-1})
5	5.27	18672	5.20	20893	6.45	20039	5.50	19345
10	10.6	9468	10.0	10337	10.9	9433	10.0	10514
20	20.2	10320	18.9	10063	21.0	9930	19.0	10282
40	40.1	6288	38.1	7358	40.4	7343	37.5	6809

Good agreement

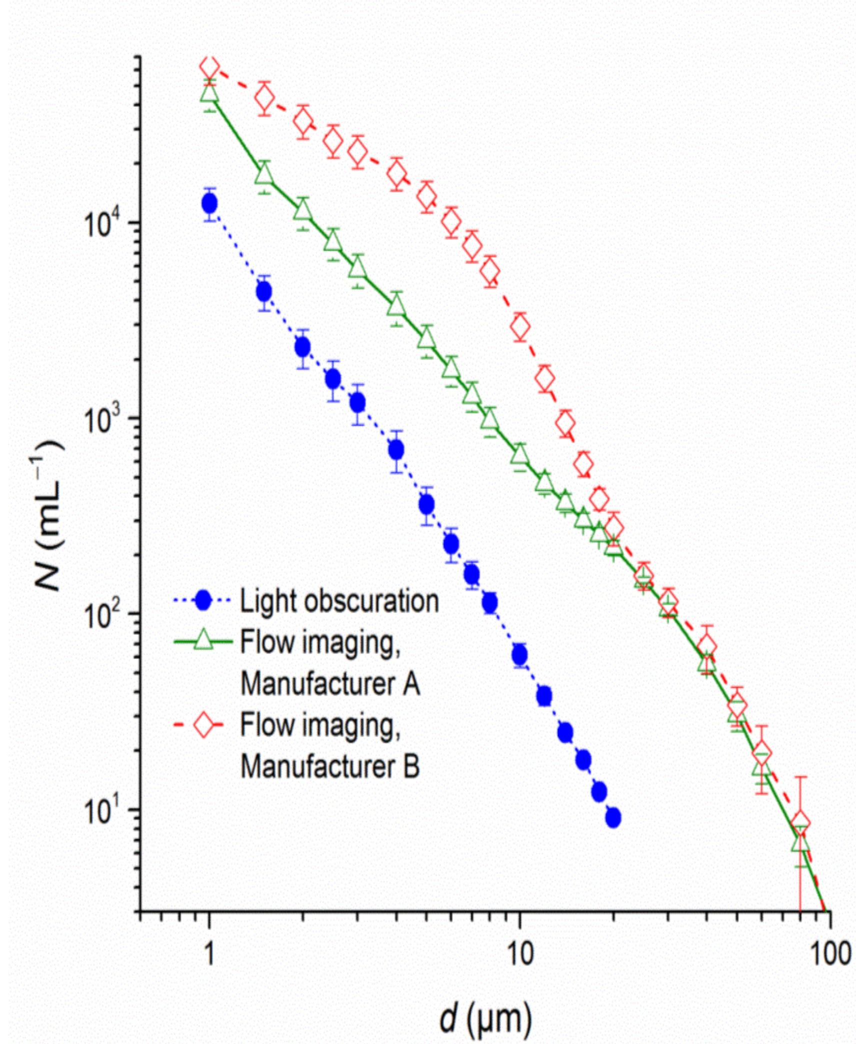
For Protein aggregates, methods don't agree…



JAMES G. BARNARD,[1] MATTHEW N. RHYNER,[2] JOHN F. CARPENTER[1]
JOURNAL OF PHARMACEUTICAL SCIENCES, VOL. 101, NO. 1, JANUARY 2012

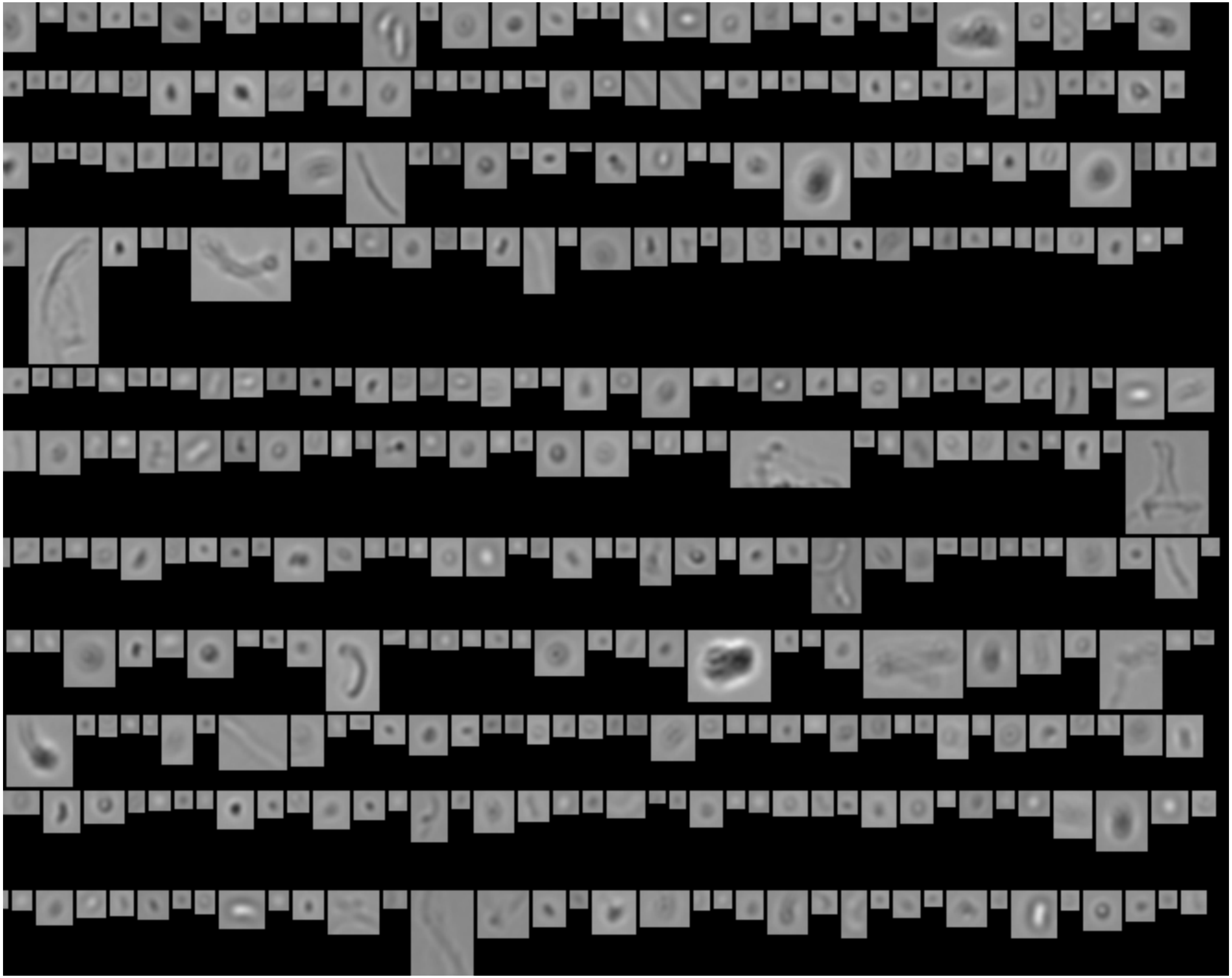


Barthélemy Demeule,[1,3] Steven Messick,[2] Steven J. Shire,[1] and Jun Liu
AAPS Journal, Vol. 12, No. 4, December 2010



Ripple, D. C. and Z. S. Hu (2016)Pharmaceutical Research **33**(3): 653-672.

Protein aggregates vary in size, shape, intensity…



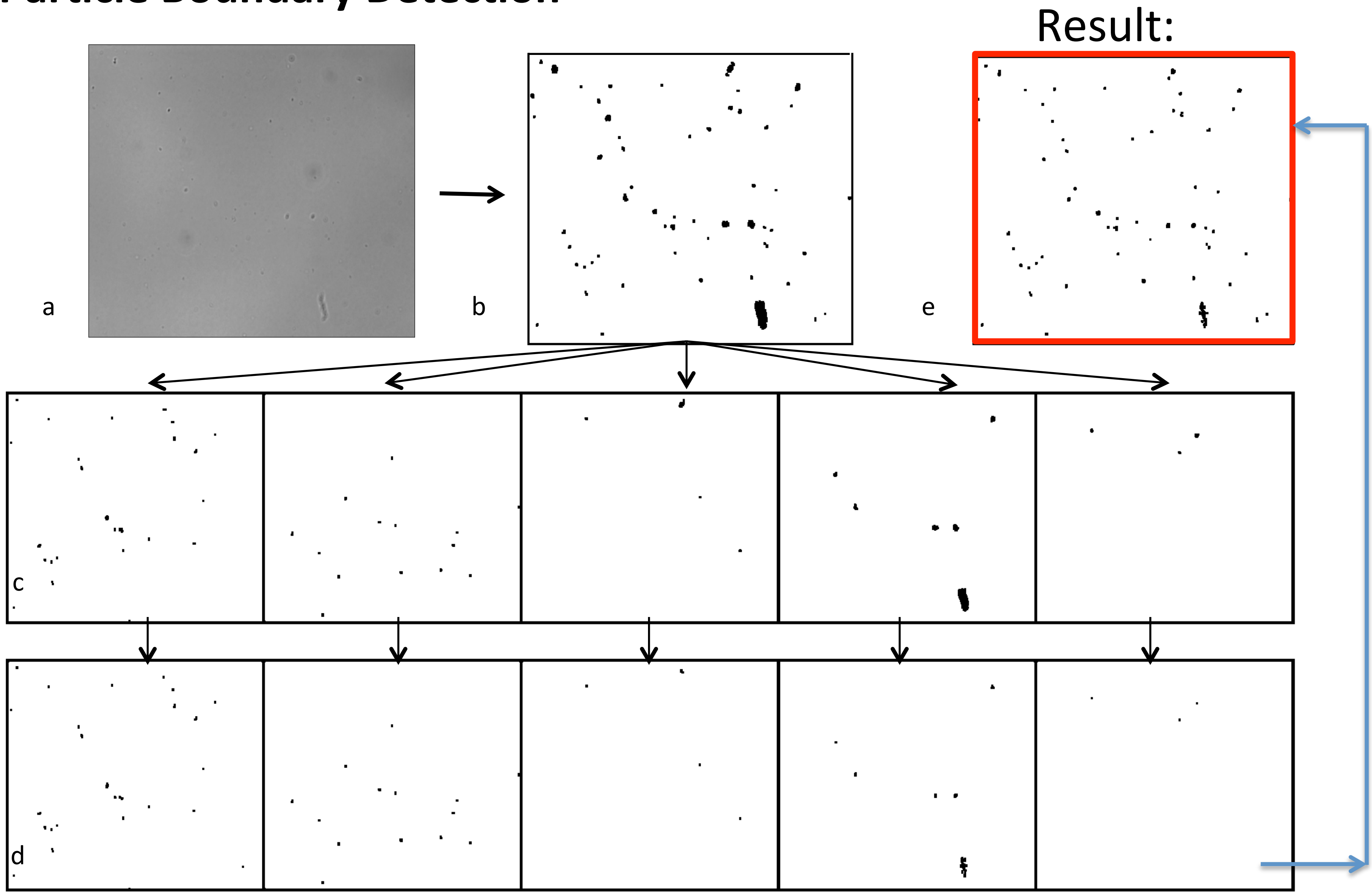
Variable Threshold Method for improved Particle Boundary Detection

Can we get improved boundary detection without losing nearly transparent particles?

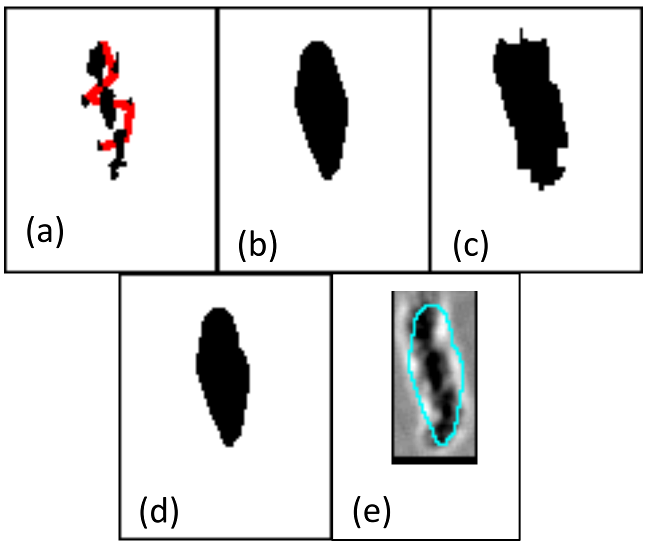
New algorithm, break analysis into two parts:

a) Find particles with a single low threshold
b) Evaluate particle boundaries using a threshold appropriate to the intensity of each individual particle

Variable Threshold Method for improved Particle Boundary Detection



Strategy for fragments



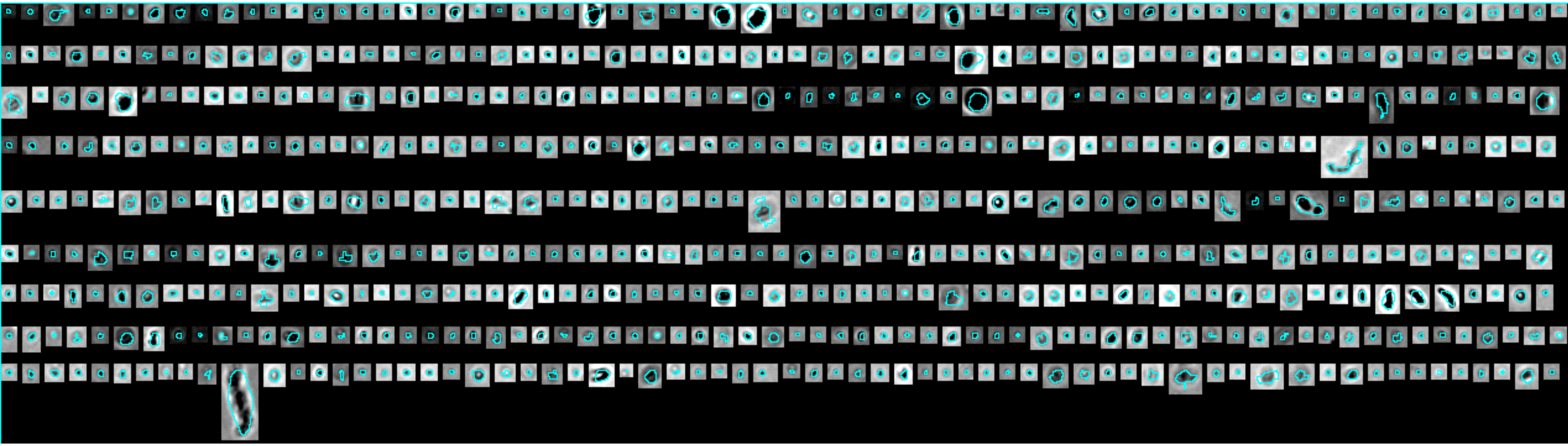
a. Connect fragments

b. Draw the convex hull

c. and d. Perform “AND” operation with original thesholded area

e. Resulting boundary superimposed on image

Collage with overlays



Results table of analyzed particles

Results

	Area	Mean	StdDev	Mode	Min	Max	X	Y	XM	YM	Perim.	BX	BY	Width	Height
1	21	224.810	11.030	233	207	237	286.262	7.929	286.177	7.873	20.142	283	5	7	6
2	16	238.875	4.288	236	232	247	779.000	8.000	779.010	7.999	13.657	777	6	4	4
3	182	231.357	10.794	227	217	255	630.209	14.797	630.186	14.816	50.042	622	8	16	14
4	23	236.000	3.542	232	230	242	357.935	13.587	357.939	13.588	16.728	355	11	6	5
5	42	206.310	1.994	205	203	210	311.667	19.167	311.667	19.173	33.456	307	14	8	11
6	302	234.371	15.702	255	197	255	681.384	32.440	681.339	32.337	103.497	674	15	16	35
7	46	240.848	2.097	241	237	245	585.674	22.500	585.676	22.494	24.385	582	19	8	8
8	18	208.722	3.250	204	204	214	398.722	23.000	398.723	23.004	14.485	396	21	5	4
9	23	194.174	1.193	195	192	196	206.587	24.978	206.597	24.985	24.971	201	22	9	7
10	16	221.500	2.477	223	217	225	598.375	24.438	598.378	24.441	14.142	596	22	5	5
11	22	179.227	3.664	180	172	186	137.591	26.591	137.594	26.583	15.899	135	24	5	5
12	8	237.750	0.886	237	237	239	627.375	26.625	627.375	26.627	9.071	626	25	3	3
13	16	202.000	2.251	203	199	206	274.250	31.000	274.252	30.994	15.314	272	28	4	6
14	13	237.231	1.301	237	235	239	387.038	32.192	387.039	32.191	13.314	385	30	4	5
15	57	194.947	18.693	194	157	233	496.342	49.254	496.504	49.234	29.556	491	45	10	8

	Major	Minor	Angle	Circ.	Feret	IntDen	Median	Skew	Kurt	%Area	RawIntDen	Slice	FeretX	FeretY	FeretAngle	MinFeret	AR	Round	Solidity
1	7.908	3.381	149.854	0.650	8.602	4721	230	-0.646	-1.252	100	4721	1	283	6	144.462	4.833	2.339	0.428	0.689
2	4.514	4.514	0.000	1.000	5.657	3822	239	0.178	-0.807	100	3822	1	777	6	135.000	4.000	1.000	1.000	1.000
3	16.653	13.915	15.847	0.913	18.028	42107	228	0.991	-0.184	100	42107	1	622	19	33.690	14.000	1.197	0.836	0.933
4	6.029	4.857	150.572	1.000	6.708	5428	236	0.069	-1.094	100	5428	1	355	12	153.435	5.000	1.241	0.806	0.885
5	9.480	5.641	96.993	0.472	11.180	8665	206	0.163	-1.014	100	8665	1	310	14	100.305	7.922	1.681	0.595	0.609
6	36.549	10.521	102.700	0.354	36.688	70780	234	-0.330	-0.807	100	70780	1	674	15	107.447	13.486	3.474	0.288	0.756
7	8.394	6.978	143.353	0.972	9.434	11079	241	0.101	-0.501	100	11079	1	582	19	147.995	7.778	1.203	0.831	0.893
8	5.282	4.339	0.000	1.000	5.831	3757	209	-0.072	-1.130	100	3757	1	396	22	149.036	4.000	1.218	0.821	0.947
9	7.763	3.772	148.294	0.464	9.849	4466	194	-0.177	-0.869	100	4466	1	201	23	156.038	5.389	2.058	0.486	0.590
10	5.179	3.933	127.411	1.000	5.831	3544	222	-0.435	-1.040	100	3544	1	597	22	120.964	4.243	1.317	0.759	0.821
11	5.554	5.043	134.999	1.000	6.403	3943	180	-0.132	-0.696	100	3943	1	136	24	128.660	5.000	1.101	0.908	0.936
12	3.538	2.879	135.000	1.000	4.243	1902	238	0.493	-1.372	100	1902	1	626	25	135.000	3.000	1.229	0.814	0.941
13	5.225	3.899	90.000	0.857	6.083	3232	203	0.036	-1.161	100	3232	1	274	28	99.462	4.000	1.340	0.746	0.842
14	4.500	3.678	101.310	0.922	5.385	3084	237	0.031	-1.053	100	3084	1	386	30	111.801	4.000	1.223	0.817	0.812
15	11.509	6.306	25.886	0.820	12.207	11112	194	-0.036	-0.572	100	11112	1	491	52	34.992	6.879	1.825	0.548	0.891

	Area	Mean	StdDev	Mode	Min	Max	X	Y	XM	YM	Perim.	BX	BY
1												283	5

Results

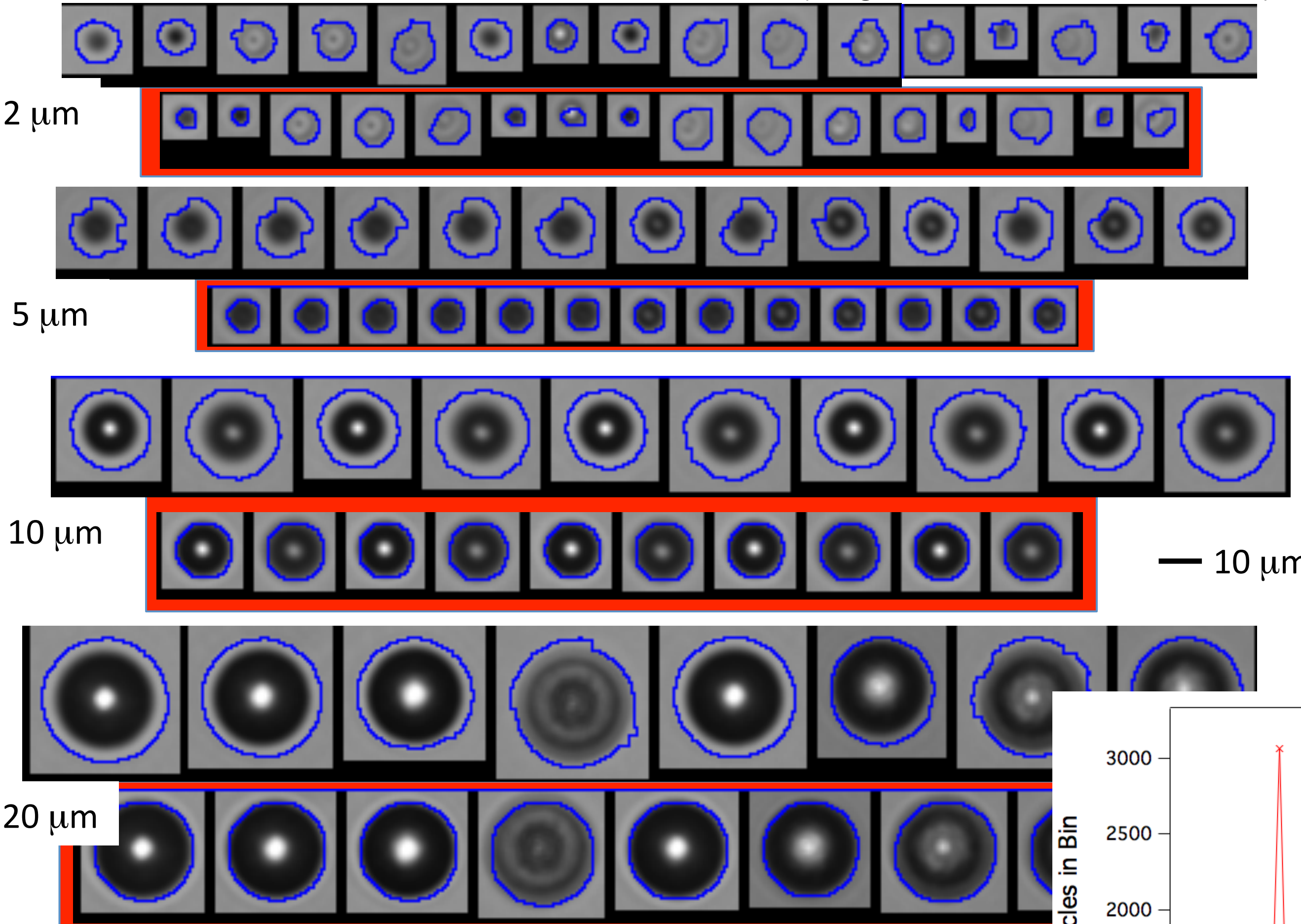
Width	Height	Major	Minor	Angle	Circ.	Feret	IntDen	Median	Skew	Kurt
7	6	7.908	3.381	149.854	0.650	8.602	4721	230	-0.646	-1.252

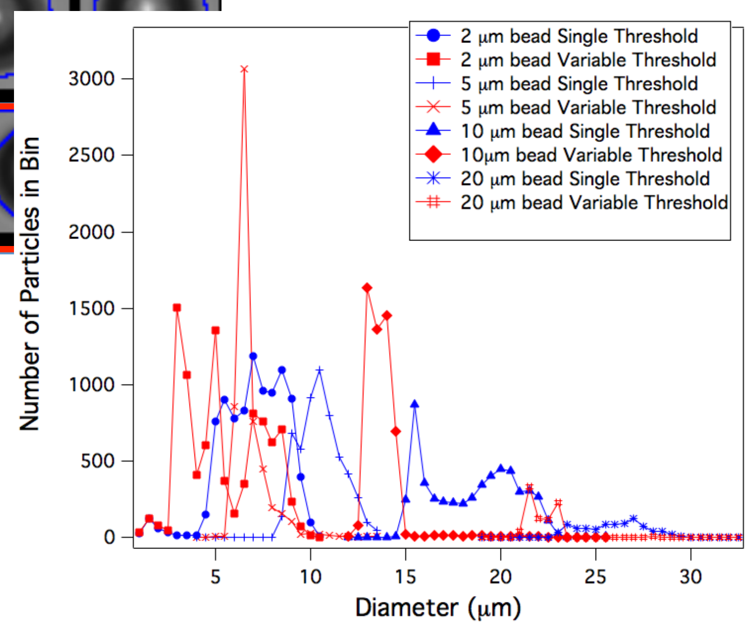
Kurt	%Area	RawIntDen	Slice	FeretX	FeretY	FeretAngle	MinFeret	AR	Round	Solidity
-1.252	100	4721	1	283	6	144.462	4.833	2.339	0.428	0.689

Implemented in ImageJ/FIJI, open source software

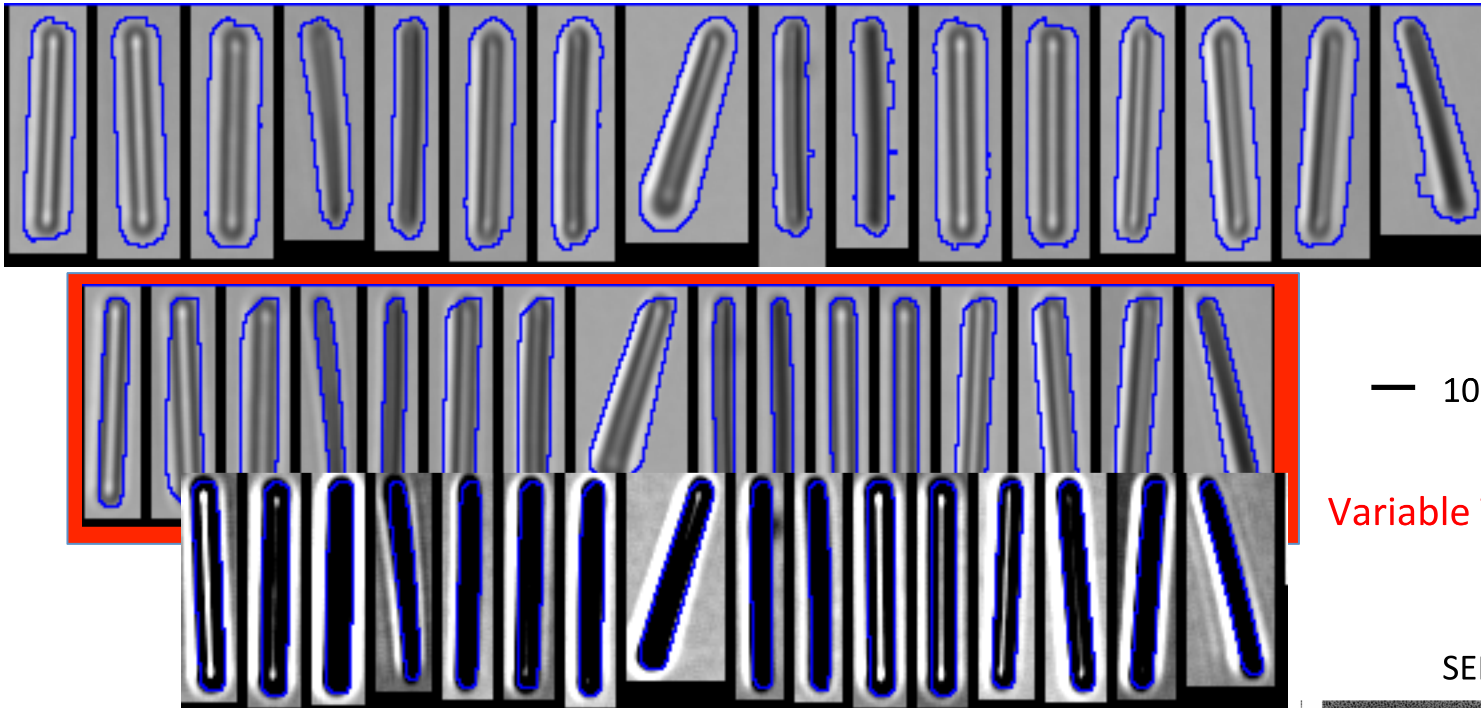
Collage strips of same beads analyzed with single threshold and Variable Threshold (red background)

(Images obtained with Flowcam 10x)



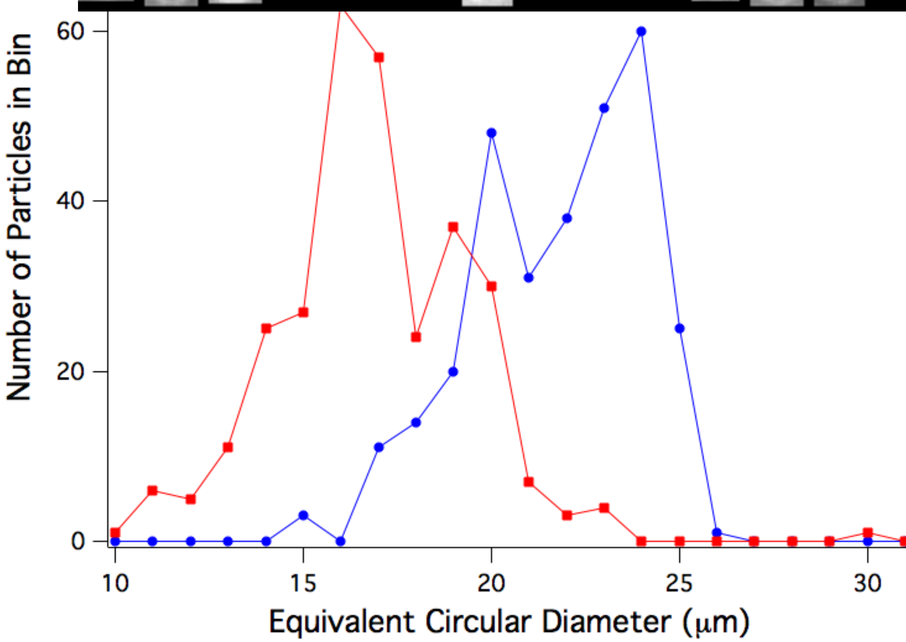


Test of Variable Threshold Method

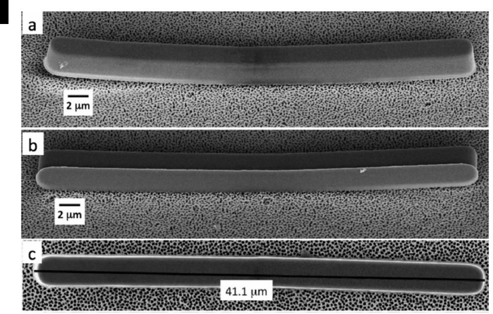


10 µm

Variable Threshold

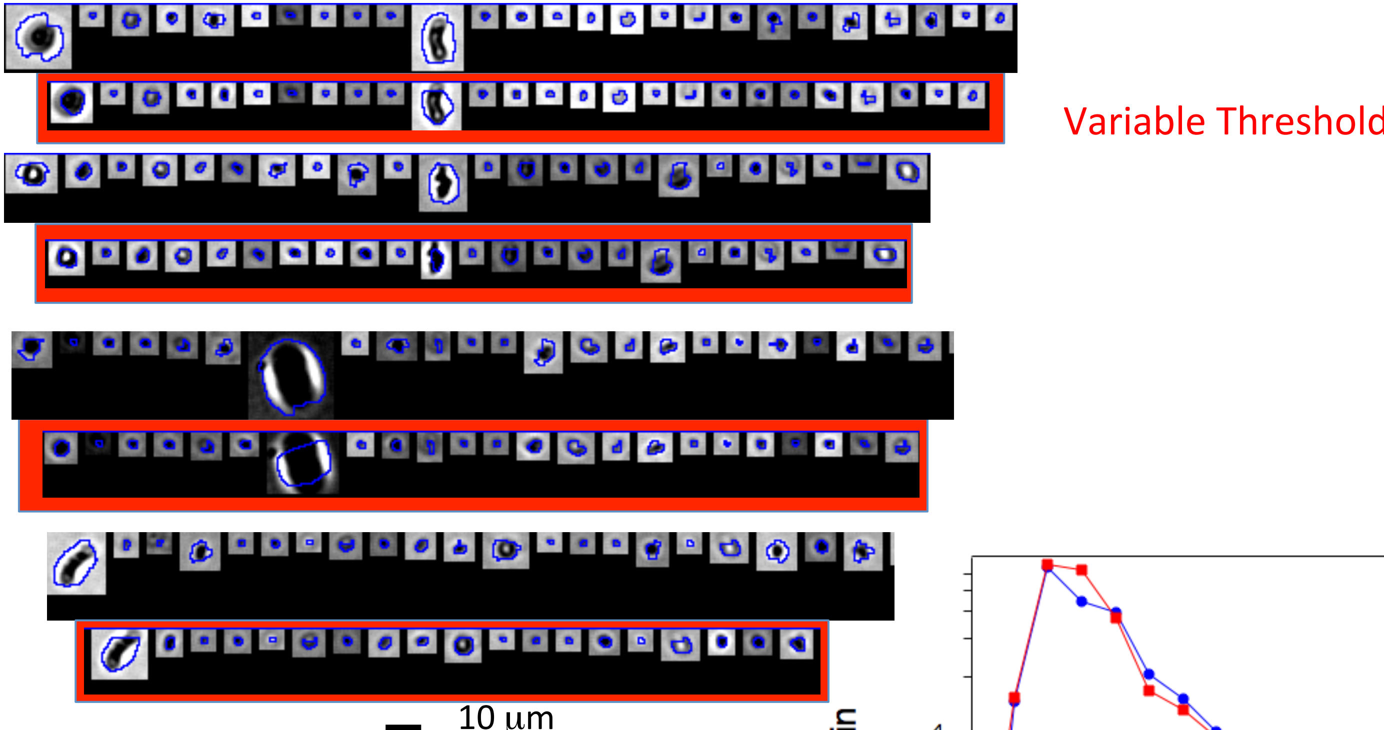


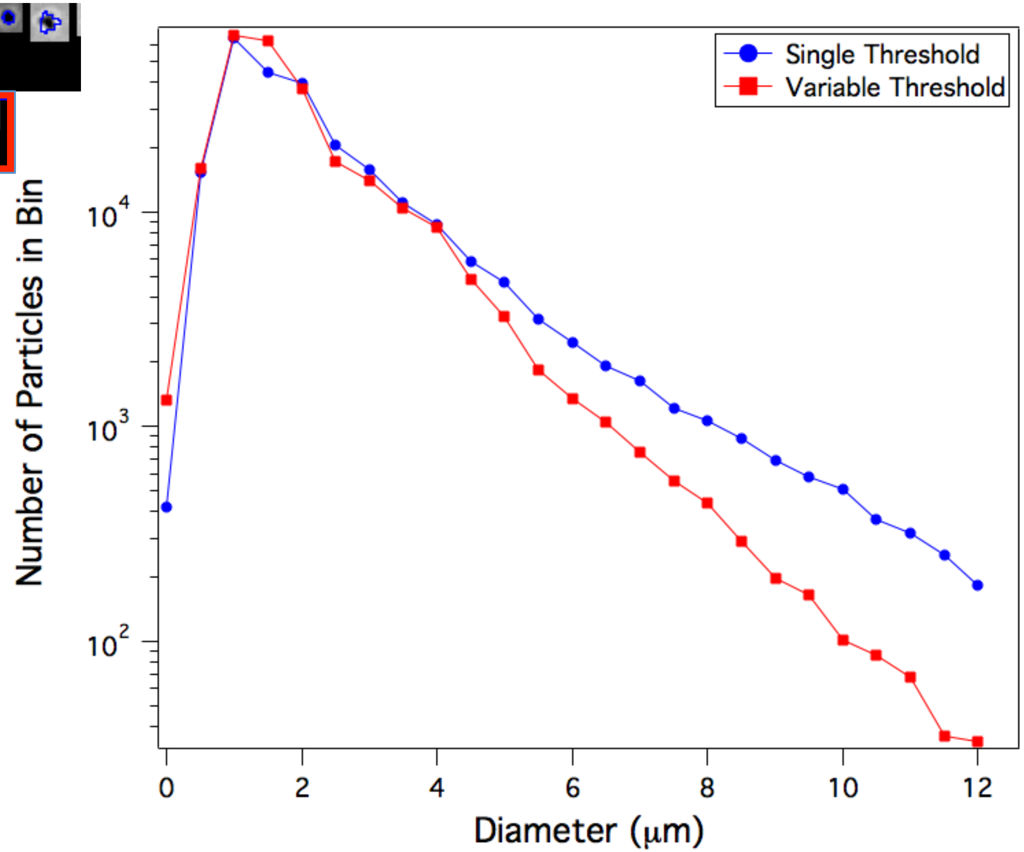
SEM image



ECD 13.4 µm

Cavicchi et al., JOURNAL OF PHARMACEUTICAL SCIENCES 104:971–987, 2015





Test of Variable Threshold Method protein aggregates

Possible improvements to Flow Microscopy Analysis

a. Shape effect, can use perimeter to improve equivalent circular diameter calculation for elongated particles.

b. Image processing algorithm yields tighter boundaries for intense particles, without losing counts on nearly transparent particles.

   i) better analysis for samples with varying particle properties

   ii) same instrument settings for calibration and operation

   iii) may be useful for improved classification of particles

What is the density of a protein aggregate and why is it important?

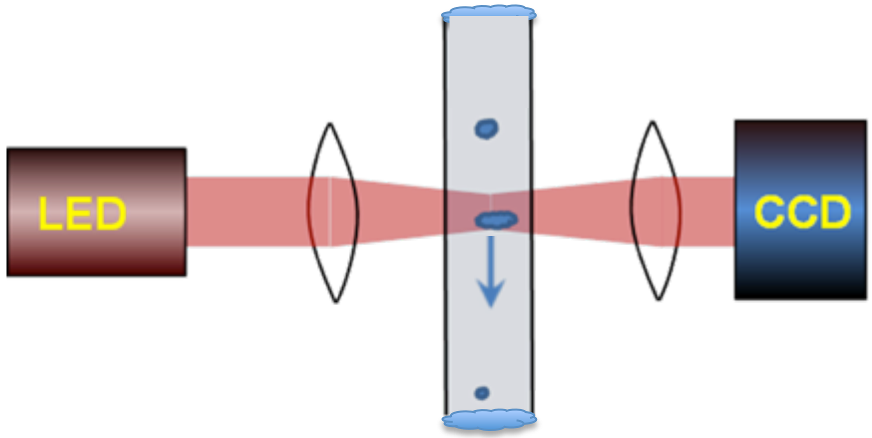
$$\rho = \frac{M}{V}$$

Different methods characterize different aspects of size:

Flow imaging: spatial extent (A~V)
Electrical Sensing Zone: liquid excluded volume (V')
Resonance Mass: M
Particle Tracking: V_h

To compare particle counts above a certain size, or size distribution from these methods, a density value for protein aggregates is needed.

Sedimentation/Particle tracking



For a sphere $\Delta\rho = \dfrac{9\mu v}{2gR^2}$

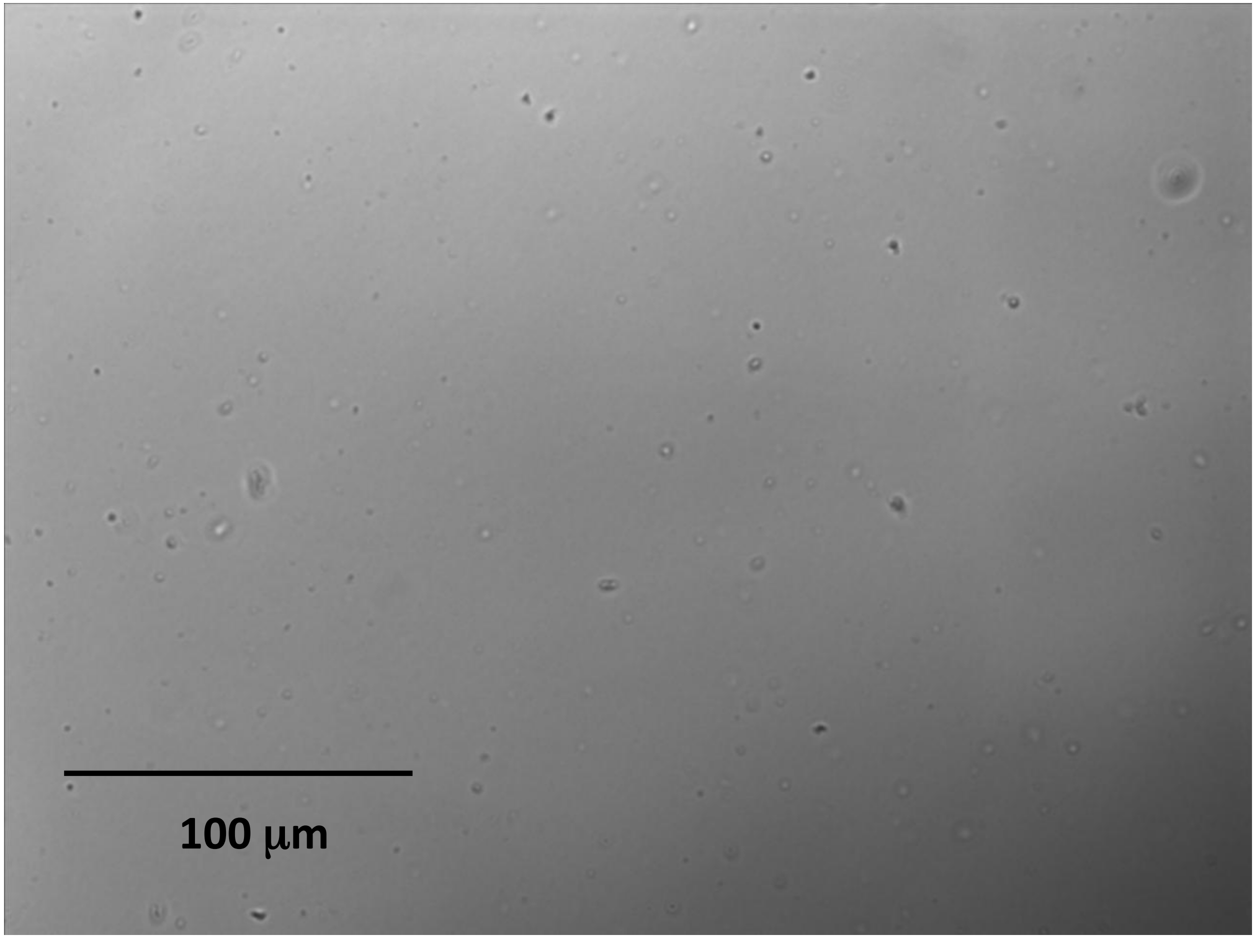
μ viscoscity
v velocity
g 9.8 m/s^2

R hydrodynamic radius of sphere

Measuring R from image, and measuring v by following images, can deduce $\Delta\rho$

To reduce convection, use Rectangular glass capillary 50 μm or 100 μm inside thickness x 1 mm, sealed at both ends

Aggregates from NIST MaB



Sedimentation: Stokes Law

$$\Delta\rho = \frac{9\mu v}{2gR^2}$$

Where μ is viscoscity, g is gravitation constant, v is the velocity (initial –final vertical position/time), and R is particle radius from image or Brownian trajectory analysis (below)

Brownian Motion- Microparticle Tracking Analysis

$$\langle x^2 \rangle = 2Dt$$

D diffusion constant

For a sphere

$$D = \frac{k_B T}{6\pi\mu R}$$

R hydrodynamic radius of sphere

$\langle x^2 \rangle$ is mean square displacement, calculated from particle trajectory (see D. Ernst & J. Kohler, Phys.Chem. Chem. Phys., 2013, 15, 845)

0.03 g/mL has been reported.[16] Because protein aggregates will contain both protein and water, their density is expected to be bounded by the density of water (1.0) and the density of the monomeric protein. Thus, we arbitrarily assumed that each particle contained (by volume) 75% of protein and 25% of water. To arrive at a mass value, the volume of a sphere for each size bin was multiplied by the protein volume fraction estimate (0.75) and the protein density value (1.43 g/mL). This mass value was multiplied by the number of particles per milliliter detected in a given size bin to give the mass per milliliter per bin values (Eq. 1). These mass estimates were then integrated over the entire particle size range to provide the integral and total mass of the sample.

What is the density of a protein aggregate?

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JOURNAL OF PHARMACEUTICAL SCIENCES, VOL. 100, NO. 2, FEBRUARY 2011

$$\text{Estimated protein mass per size bin} = (0.75) \times (\text{Volume}) \times (1.43\ \text{g/mL}) \times (\text{Number of particles}) \quad (2)$$

DOI 10.1002/jps.24156

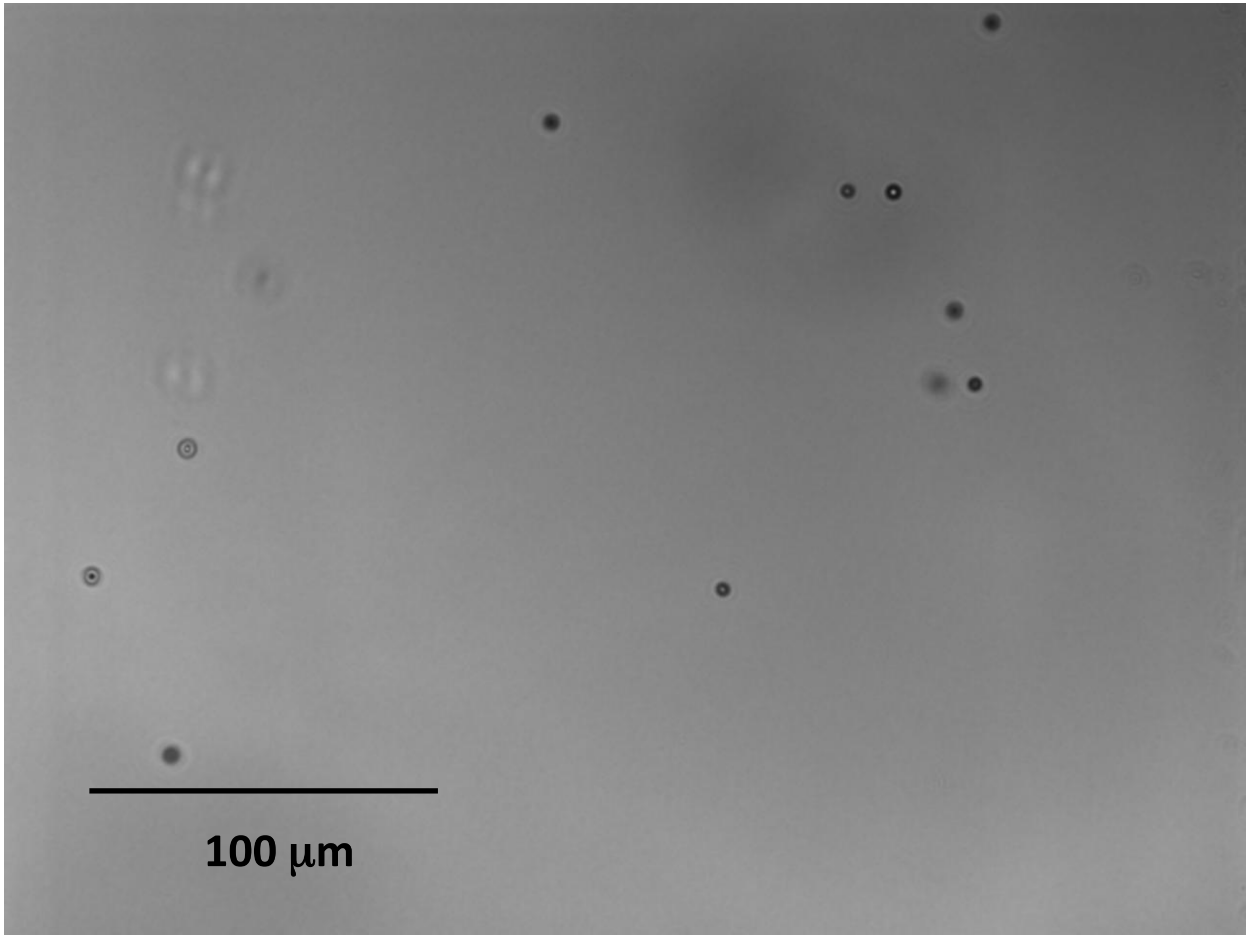
...ements from MFI can be further used to develop mathematical models to improve calculated estimates of particle volume, surface area, and mass.

In the Barnard et al. method,[17] it was assumed that protein particles were spherical in nature and that particle density was composed of 75% protein and 25% water. A protein density of 1.43 g/mL (determined by Quillin and Matthews)[37] was also used in their calculations. For our method, we not only used MFI data to account for the nonspherical nature of the particles, we also implemented some updated assumptions about the composition of protein particles. First, we used a mAb density value of 1.41 g/mL. This value was obtained using an equation developed by Fischer et al. that accounts for the empirical dependency of protein density on its molecular weight.[40] Second, we estimated the volume fraction of protein in a protein particle to be 0.2 based on several considerations: (1) protein crystals have large interstitial volumes that are around 50% of the total volume[42]; (2) using an RI of 1.41[28] and dn/dc of 0.19 mL/g[43] at 589 nm for protein particles, we can estimate the concentration of protein in a particle suspended in water to be ~0.4 g/mL (~28% protein volume fraction) because the relationship has been shown to be linear for other proteins at much greater concentrations[44,45]; and (3) the density of IgG layers adsorbed to different surfaces was measured to be 1.05–1.10 g/mL,[46] which corresponds to volume fractions of ~10%–24% in pure water. By assuming 0.2 to be the volume fraction of protein in a particle, the protein mass values calculated using the Barnard et al. method[17] are reduced by ~3.75-fold. It would be beneficial in the future to develop an analytical method to determine the average volume fraction of protein contained within particles generated from different stresses to further improve the accuracy of these particle mass calculation methods.

The particle mass calculation used in this work is referred to as the E-V method. On the basis of observation that subvisible particles tend to range from fiber like to roughly spherical,

Kalonia et al., JOURNAL OF PHARMACEUTICAL SCIENCES 104:536–547, 2015

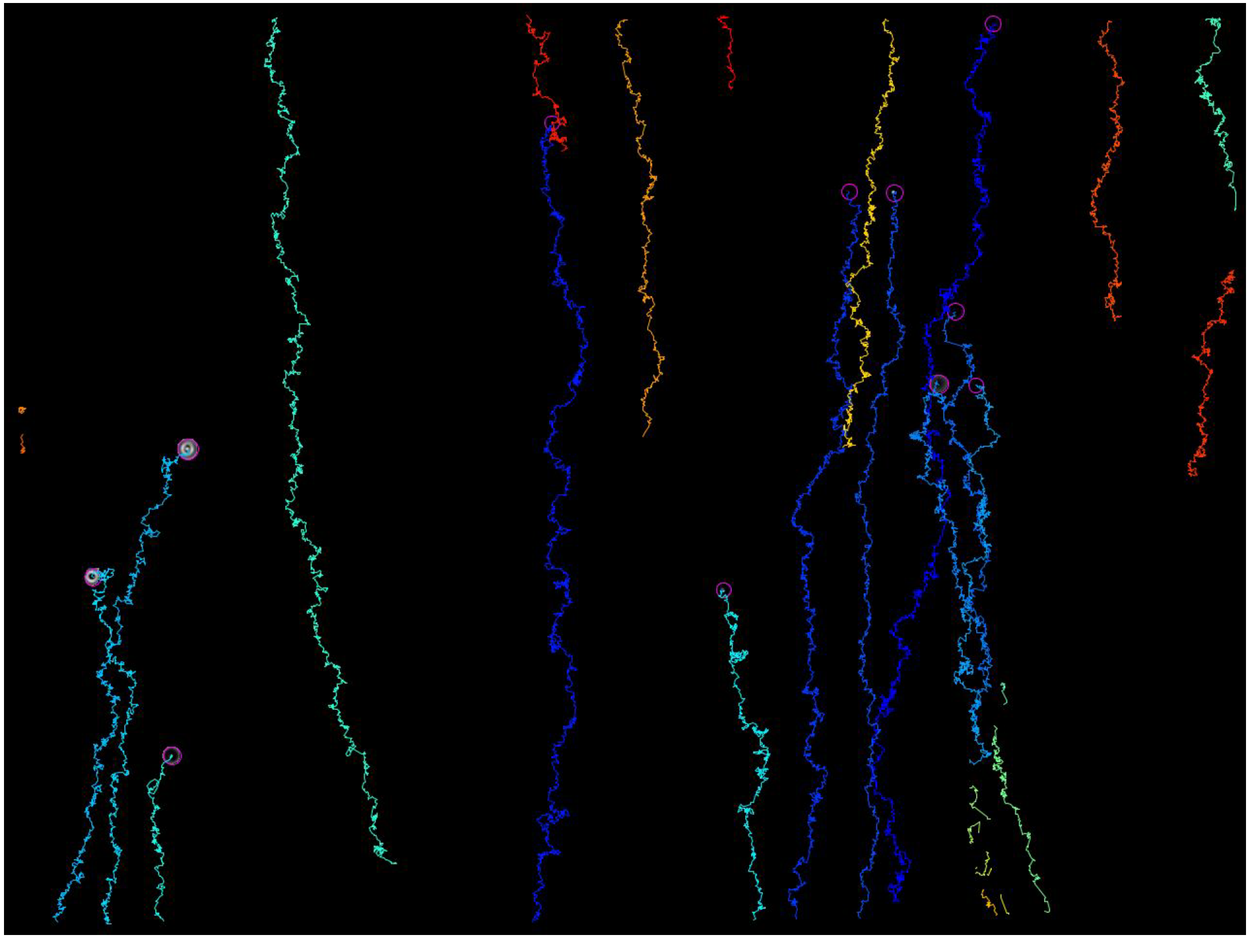
3 mm Polystyrene beads in water



3 mm Polystyrene beads in water, background subtracted

100 μm

3 mm Polystyrene beads in water, with tracks

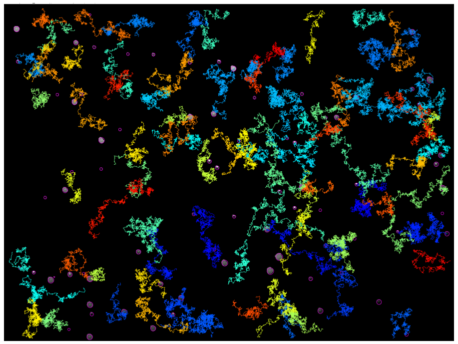


100 μm

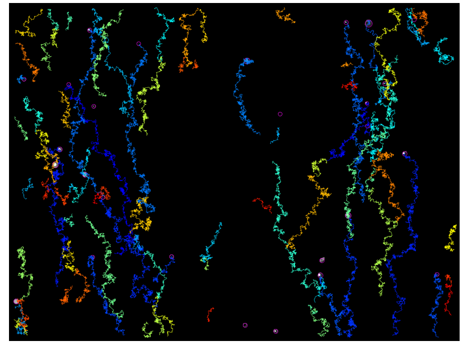
Particles analyzed by custom ImageJ plugin
Tracks determined using Trackmate

Bead trajectories - Microparticle tracking analysis

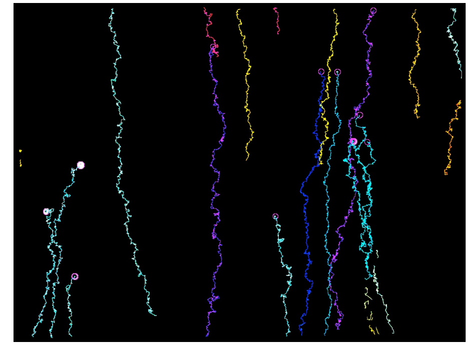
1 µm



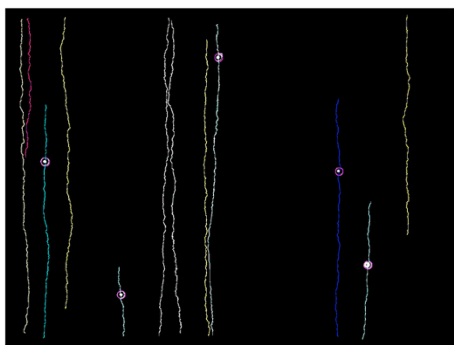
2 µm



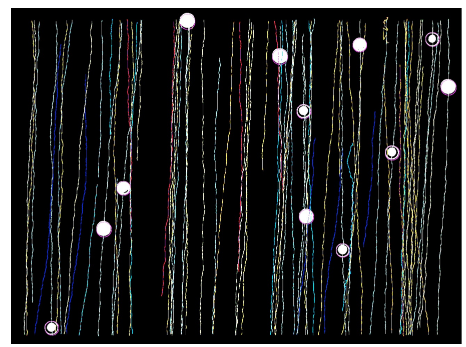
3 µm



5 µm

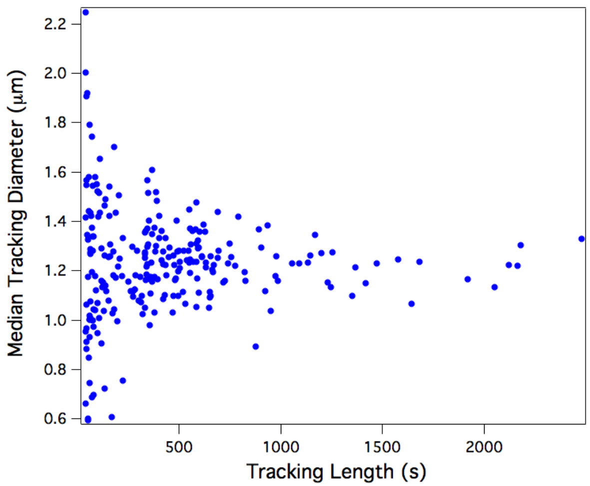


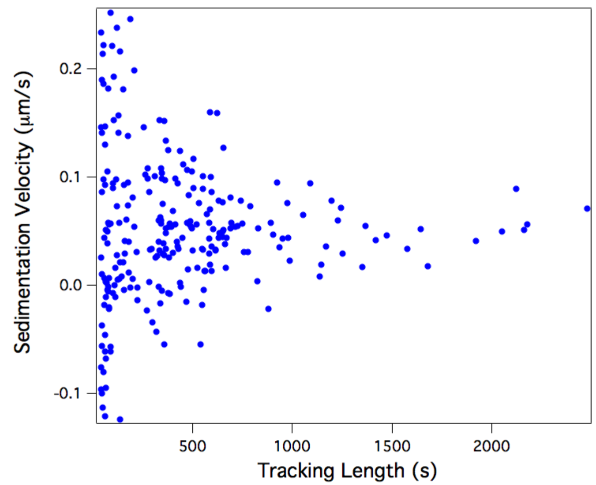
10 µm

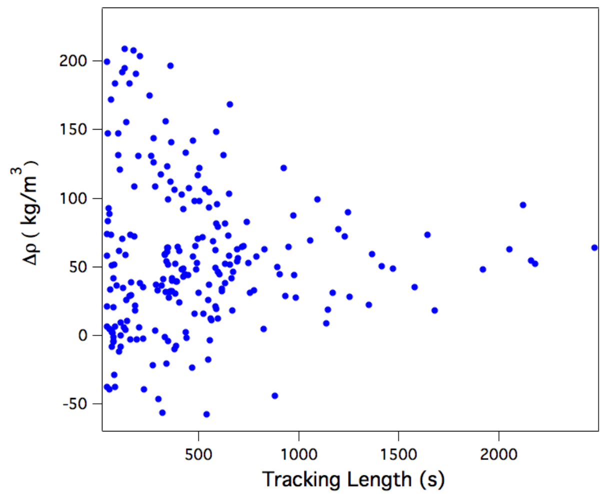


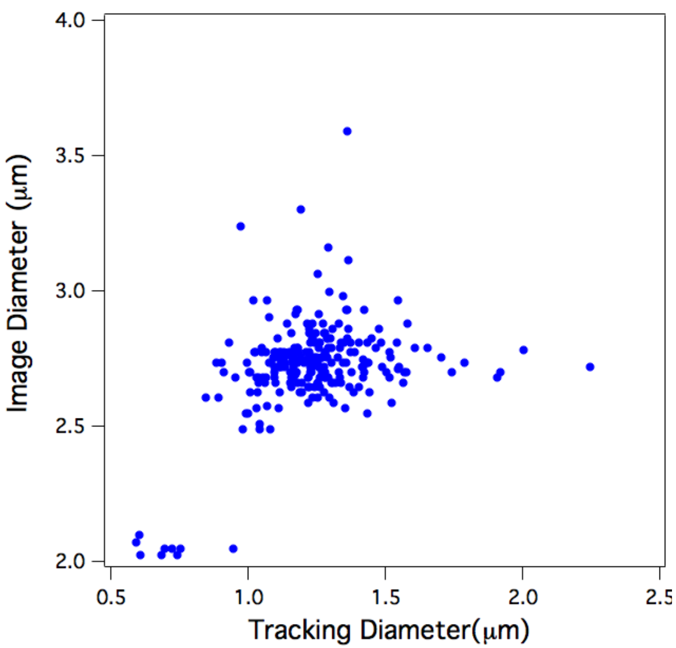
Polystyrene beads in water

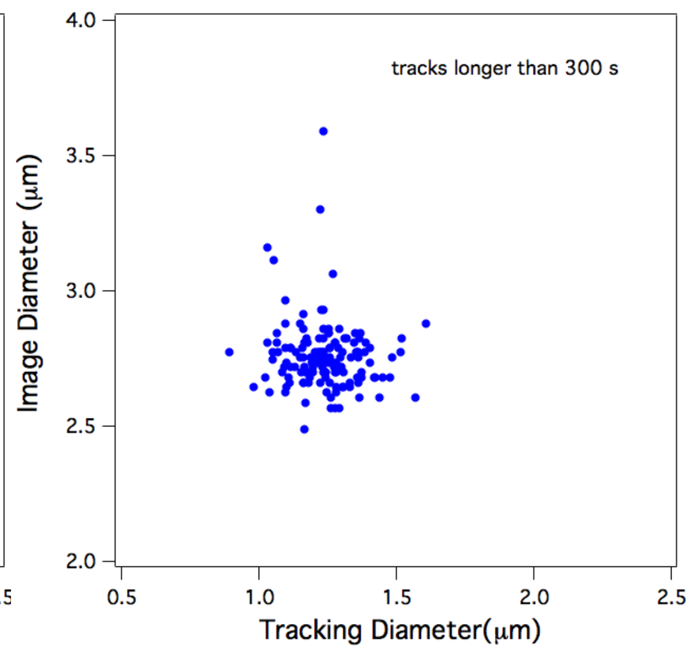
Results 1 μm bead



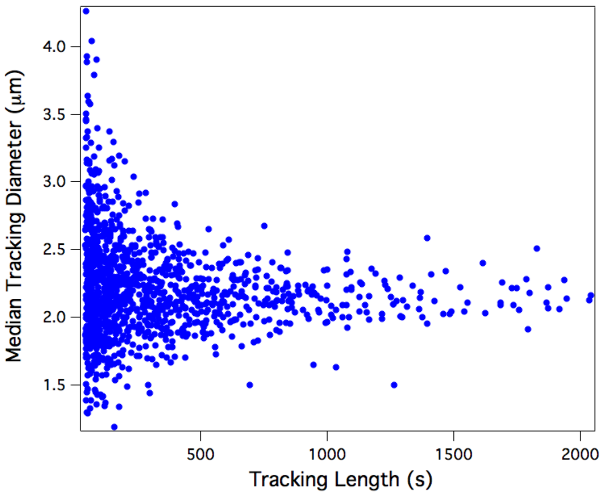


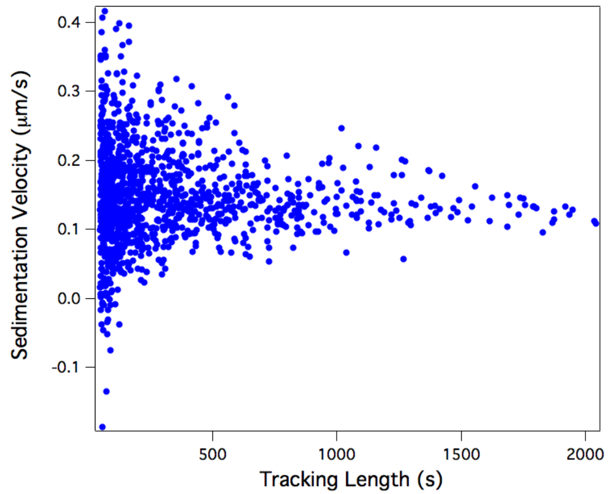


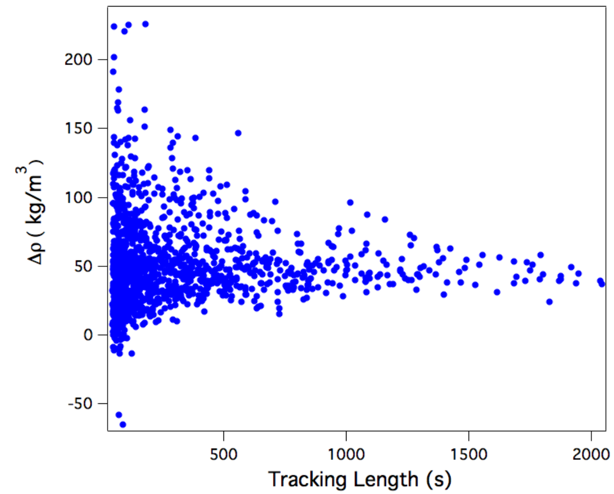


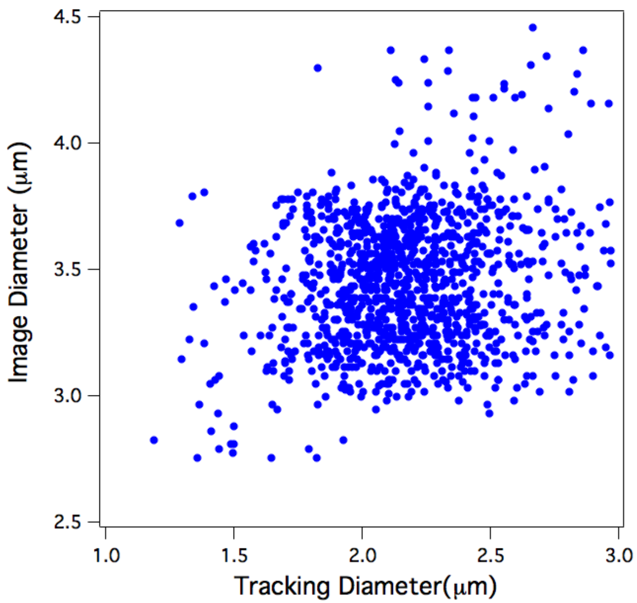


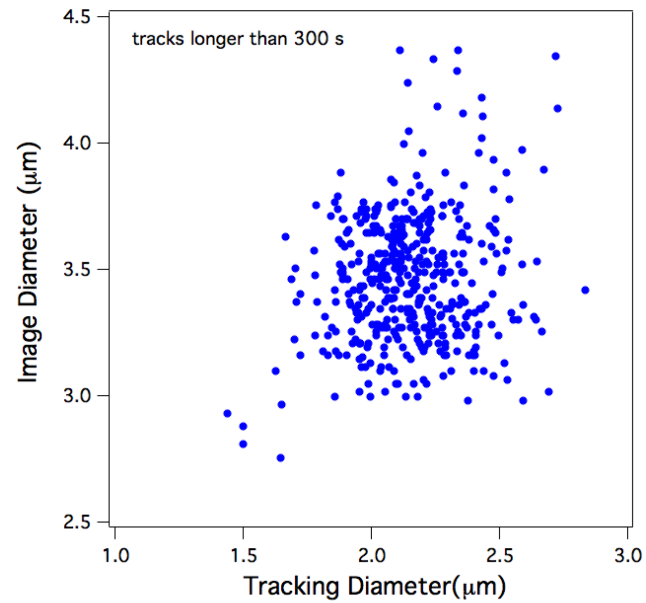
Results 2 μm bead



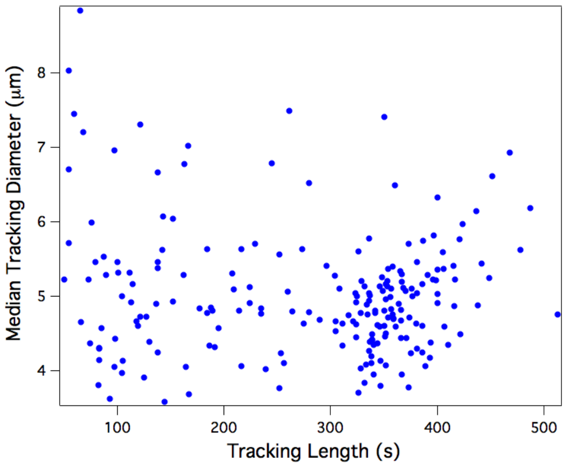


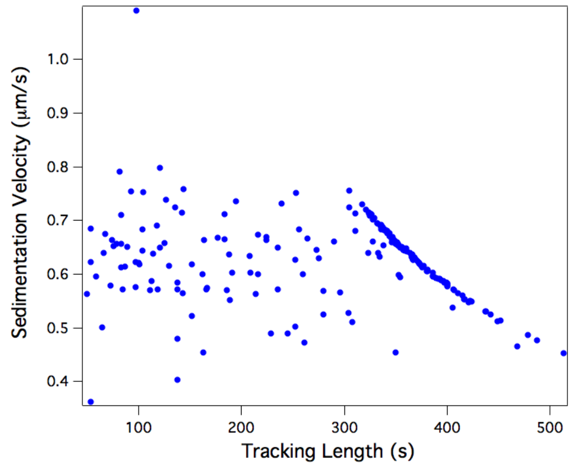


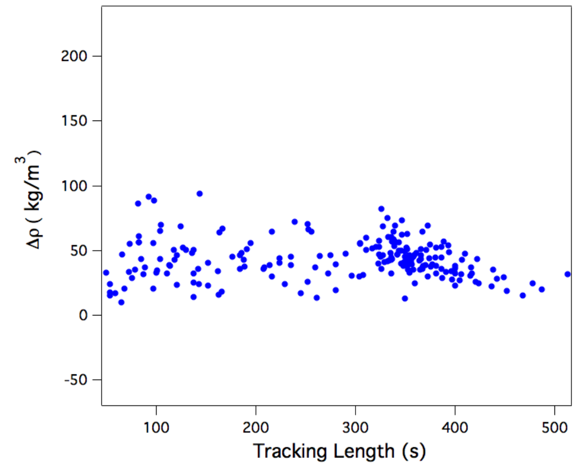


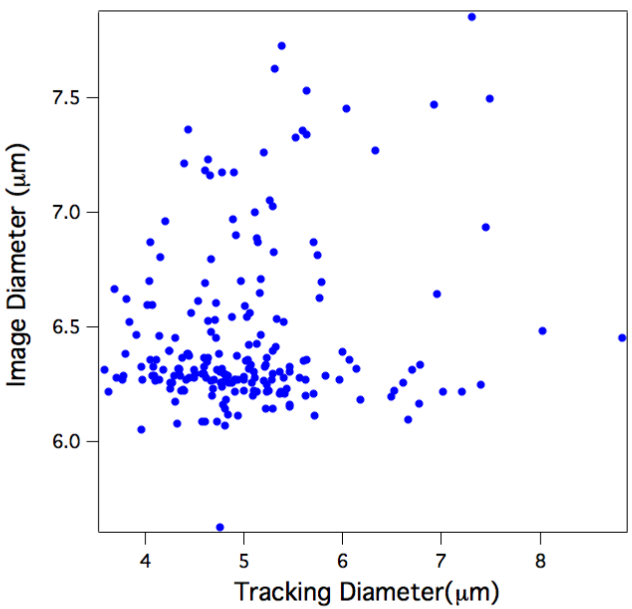


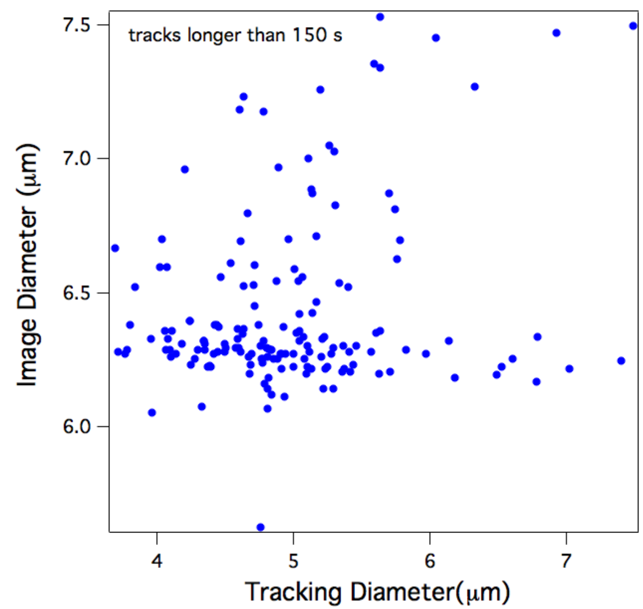
Results 5 μm bead



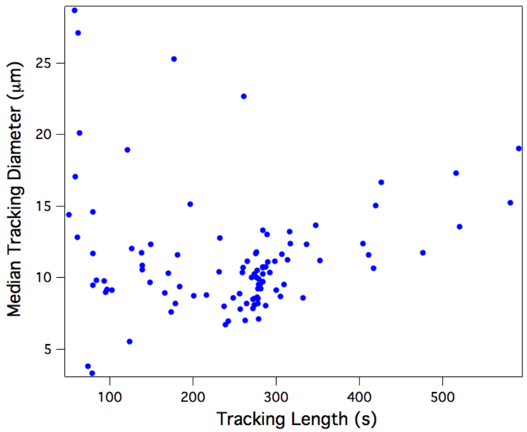


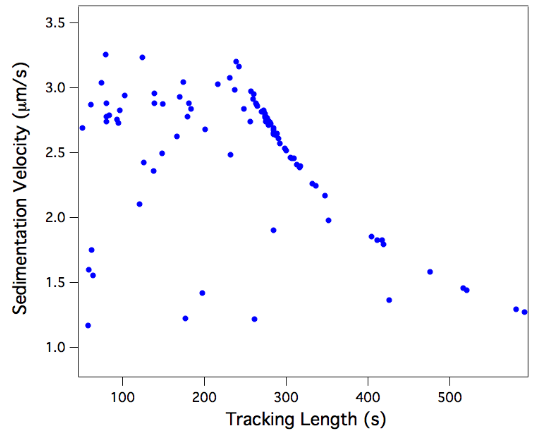


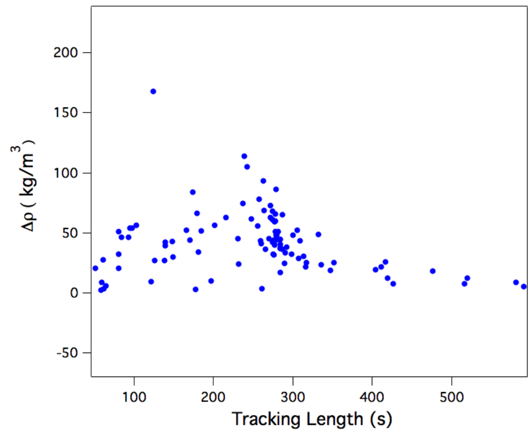


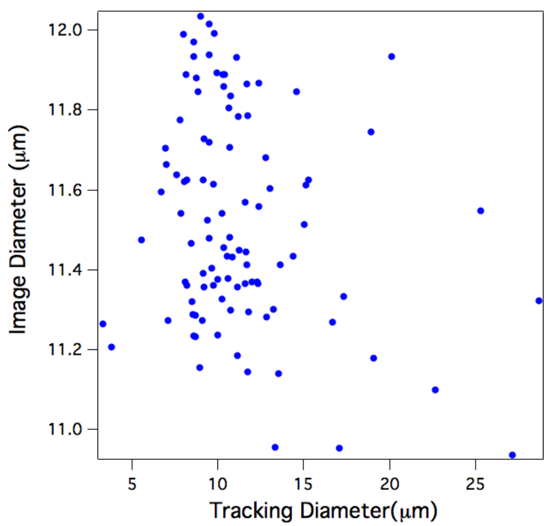


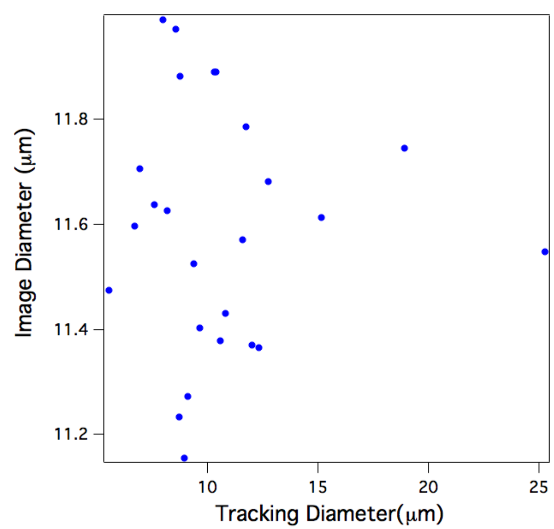
Results 10 μm bead



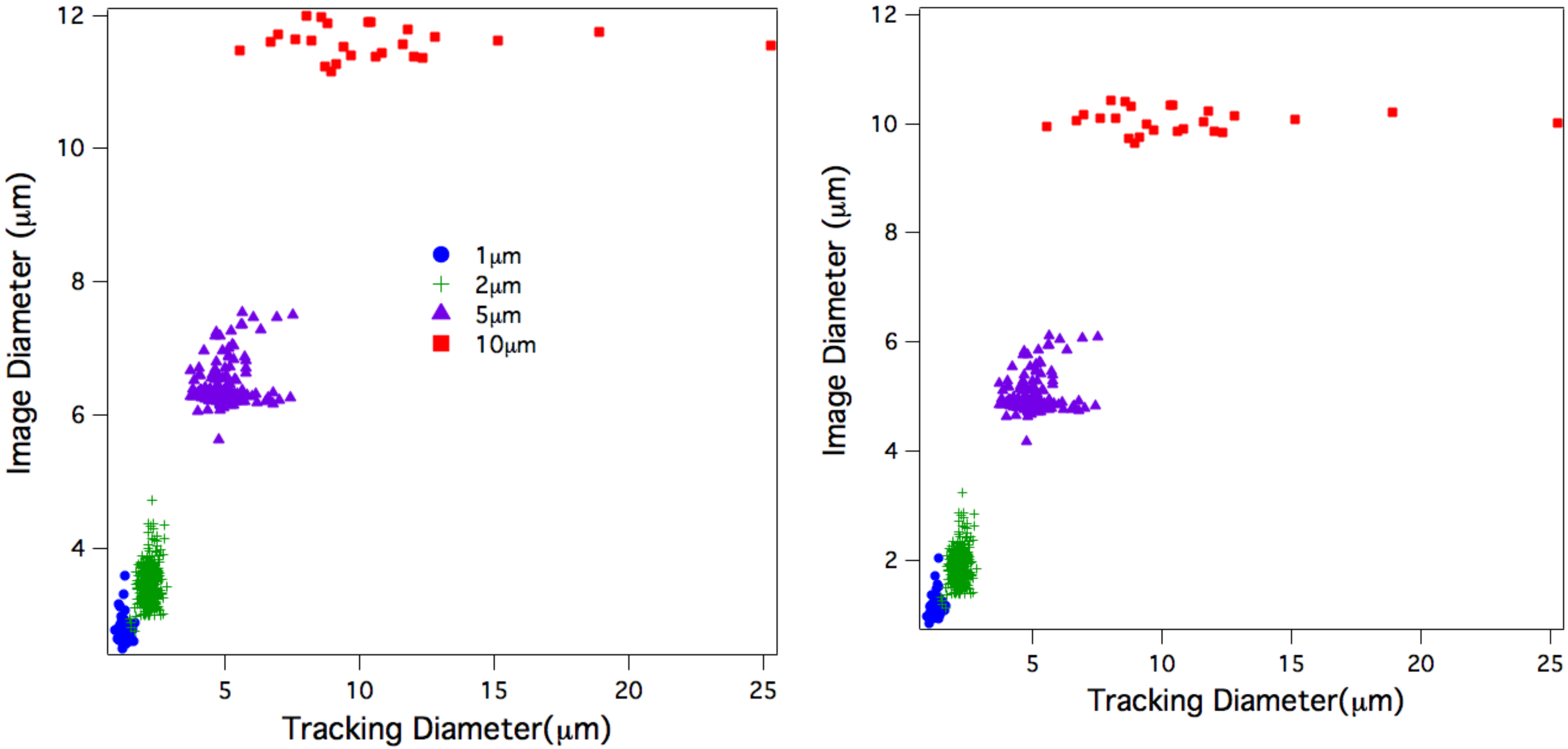








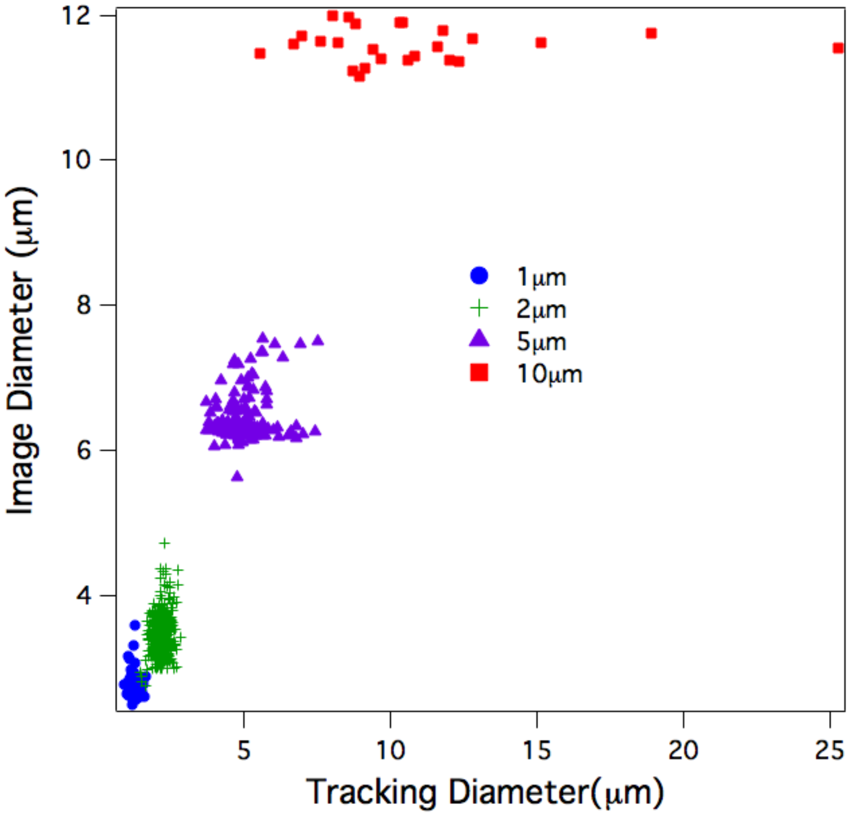
Beads: Image Diameter vs Tracking Diameter



Beads: Density by MSD & Image(after diam correction)

	MSD	Image
1	54.2431	66.7852
2	51.9681	69.5542
5	43.1026	41.0793
10	53.313	43.664

Beads: Image Diameter vs Tracking Diameter



Beads: Density by MSD & Image

	MSD	Image
1	54.2431	66.7852
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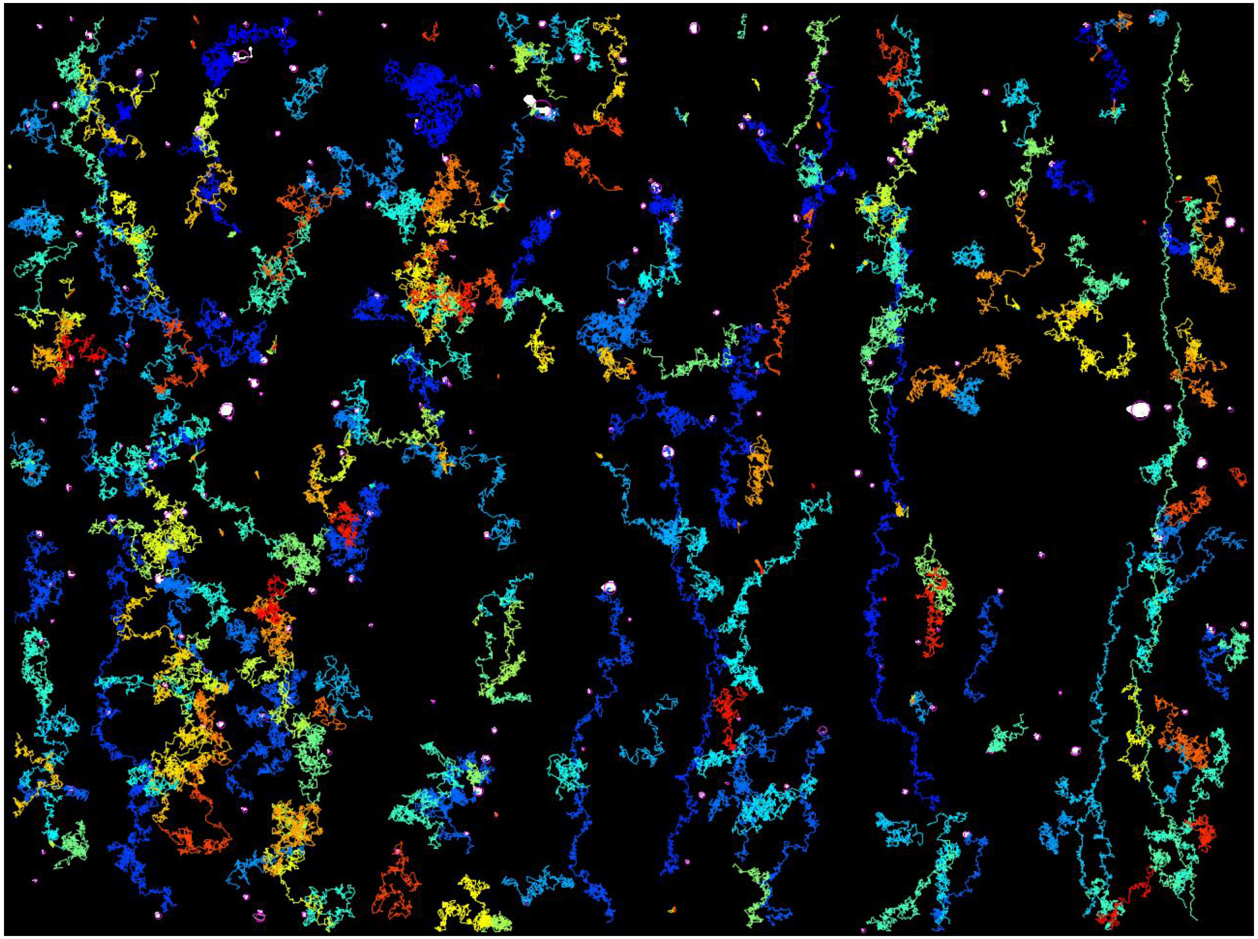
5	43.1026	41.0793
10	53.313	43.664

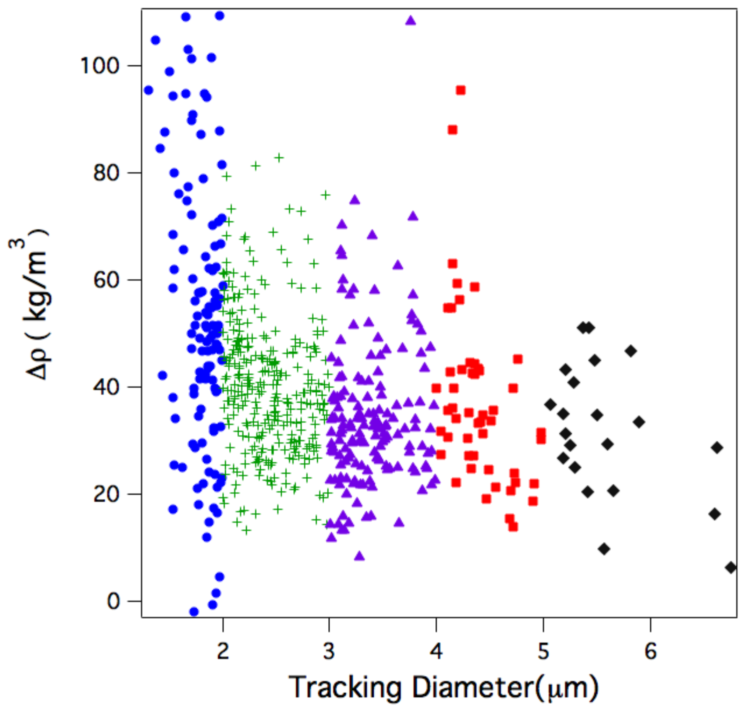
Aggregates from NIST MaB with tracks



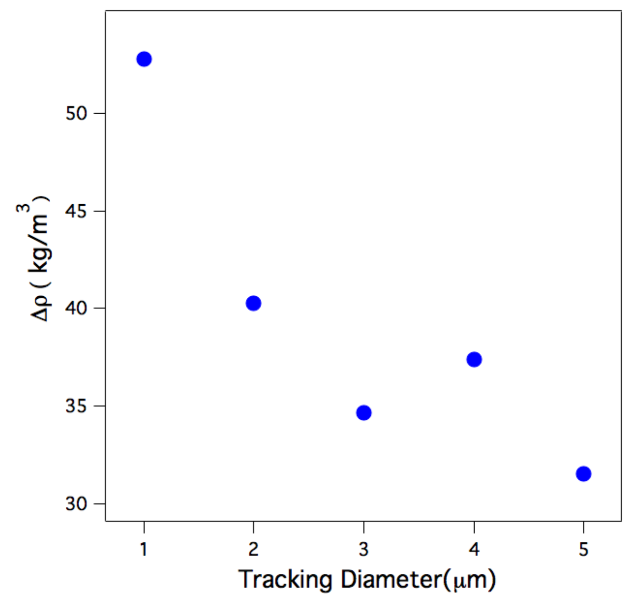
100 μm

Particles analyzed by custom ImageJ plugin
Tracks determined using Trackmate

Density vs Tracking Diameter



Average



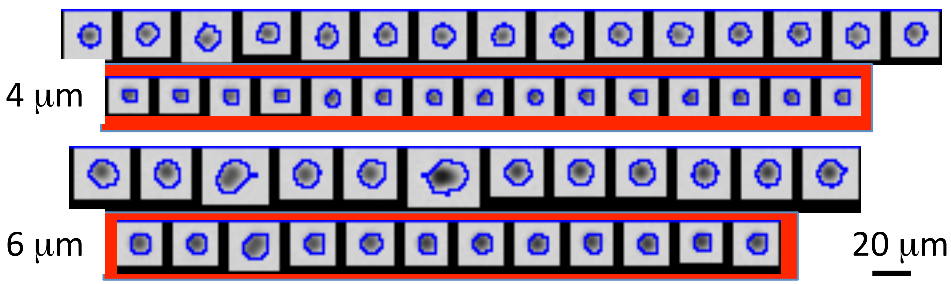
Avg density ~1040 kg/m^3

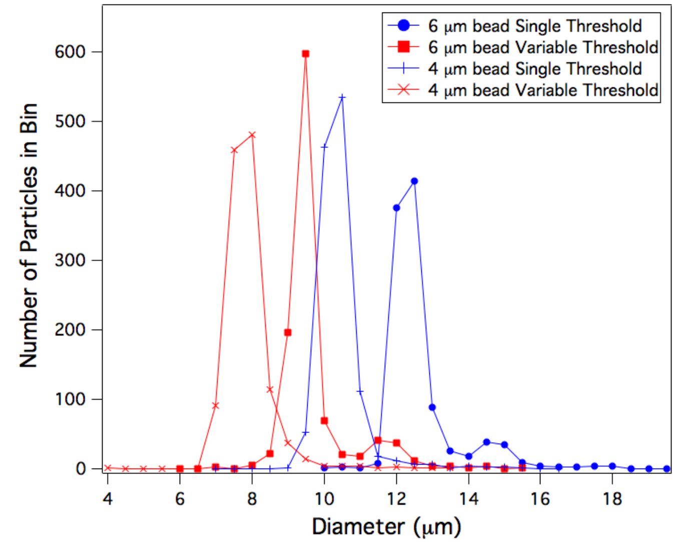
Aggregate density increases with decreasing size

Conclusions

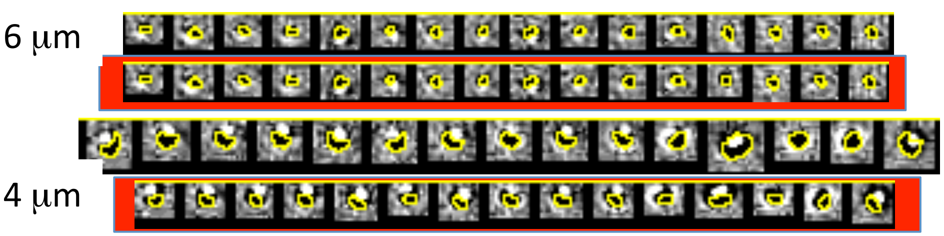
- Improvements in analysis can provide better accuracy in particle sizing for Flow imaging: variable threshold plugin for ImageJ/FIJI
- Particle density critical for relating counts from different instruments
- Microparticle tracking gives useful dimensions up to ~5 μm
- Results based on sedimentation of 1μm-7 μm particles indicates a density of 1040 kg/m^3, with density increasing with decreasing size. This density is lower than generally assumed in the literature.

Silica beads n=1.33 fluid

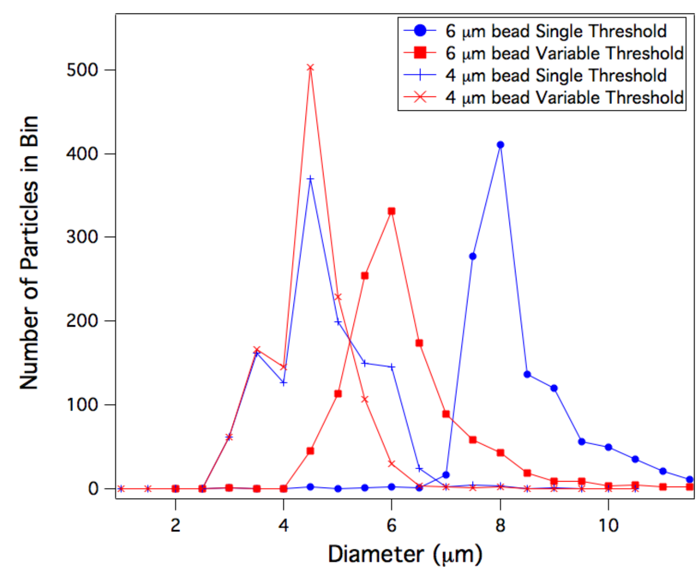




Silica beads n=1.43 fluid



Variable Threshold



Test of Variable Threshold Method

Protein aggregate at different focal distances