

Extending Exposure Assessment Strategies for Respirable Fibrous Materials to the Nanoscale

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Status of WHO Fibre Exposure Assessment

- Due to their **high toxicological relevance**, for asbestos and other **biopersistent** WHO fibres **number-based** occupational exposure levels in the order of $10\,000\text{ F/m}^3$ have been established.
- However, for **historic reasons** of epidemiologic dose comparability to PCM, also contemporary EM-based counting rules generally **only count** WHO fibres **thicker** than **200 nm**.
- Thus, **established counting rules** are **not applicable** to **nanoscale** fibres.

As a consequence ...

- Still **too little is known** about the toxicology of **nanoscale asbestos** fractions and of the many other man-made **nanoscale fibres**.

To motivate the following, let's ask ...

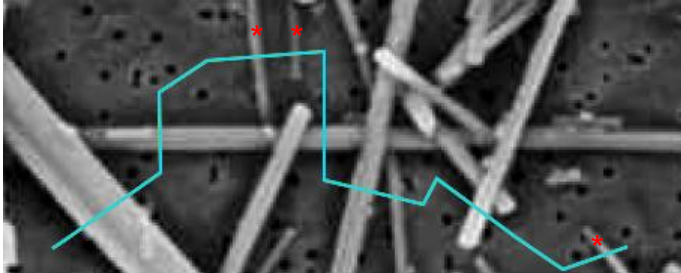
1. Are there nanoscale asbestos fractions?
2. How big is the world of engineered/advanced nanofibres?
3. Show biopersistent nanofibres fibre toxicity?

1. Are there **nanoscale** asbestos fractions?

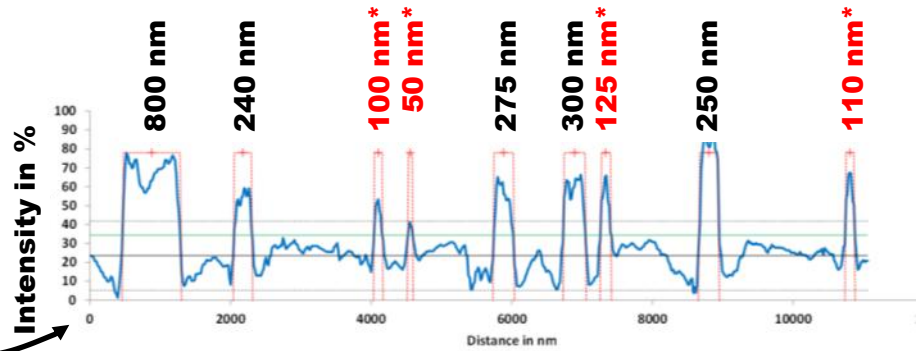
thinner than 200 nm that remain currently uncounted*

*DGUV 213-546

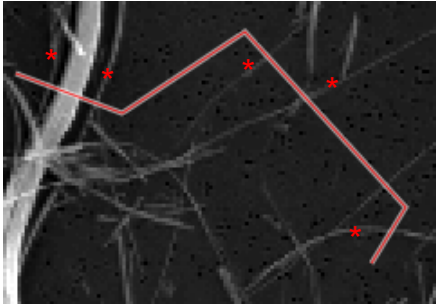
Krokydolith



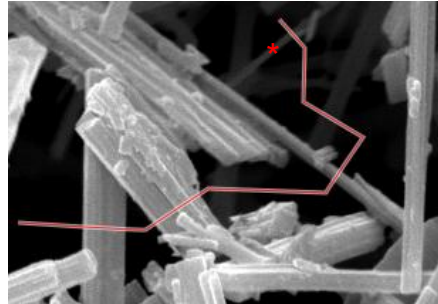
[Creutzenberg, O., et al. (2005). Schriftenreihe der BAuA.]



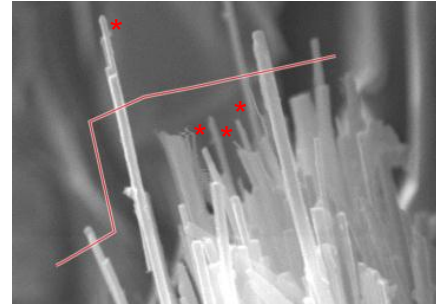
Chrysotile



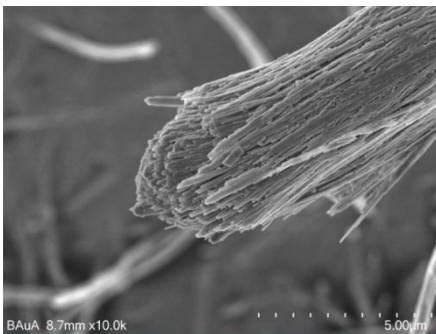
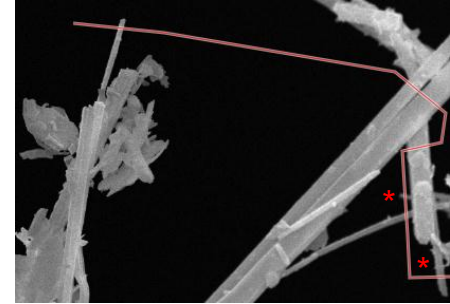
Amosite



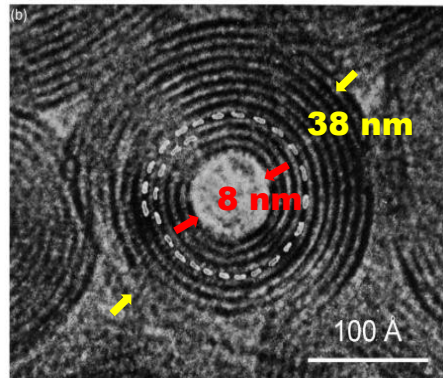
Winchite-Richterite



Anthophyllite



BAuA 8.7mm x10.0k



[Lotz, B. and S. Z. D. Cheng (2005). Polymer 46(3): 577-610.]



Yes ✓

Chrysotile is a bundled nanoscroll !

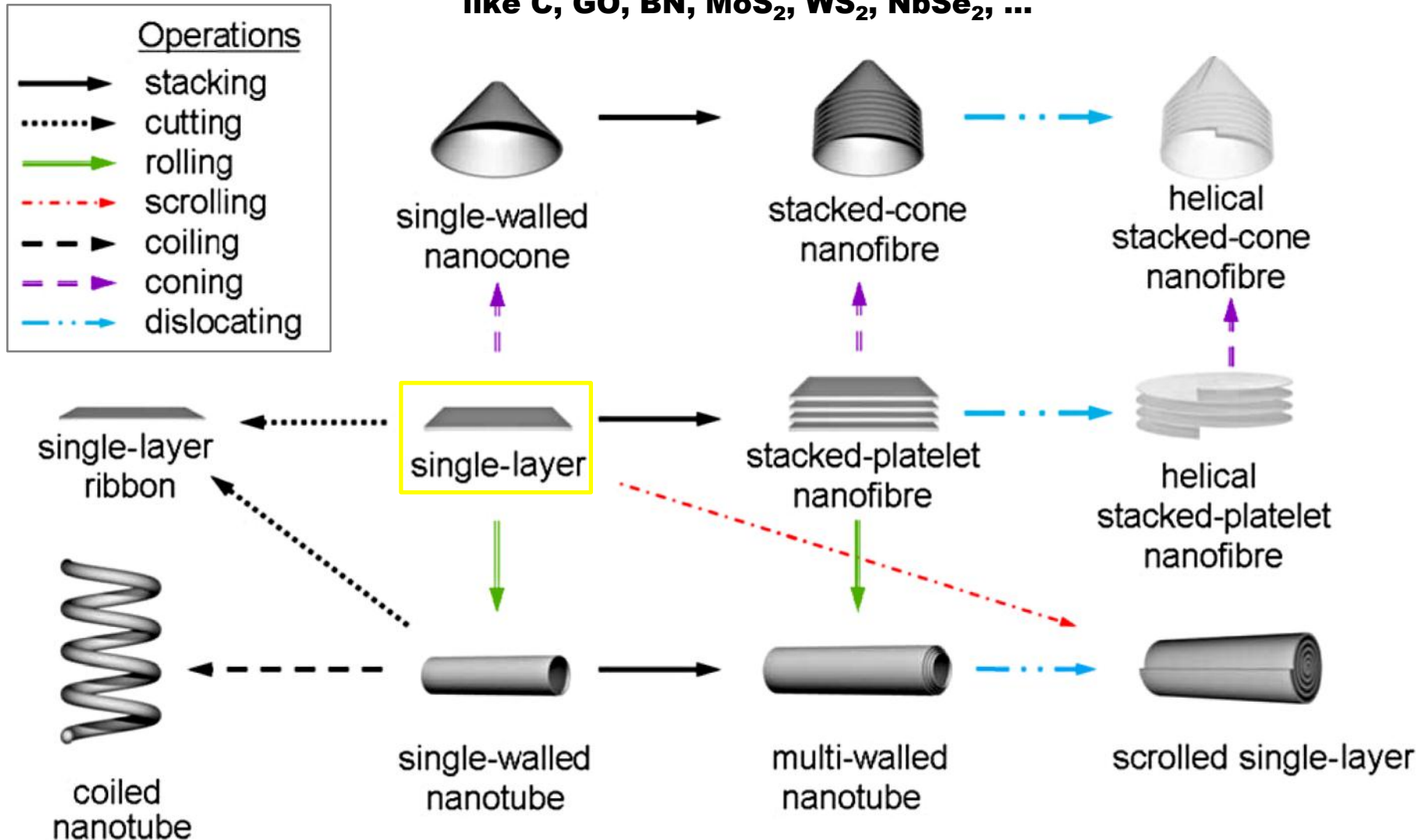
Brucite 2-layer system with internal stress but finite flexural rigidity

Dissolving or undetected after de-bundling?

2. How big is the world of advanced fibres?

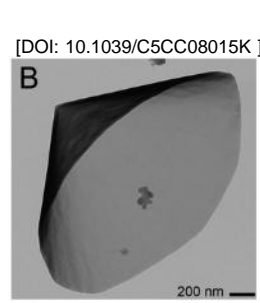
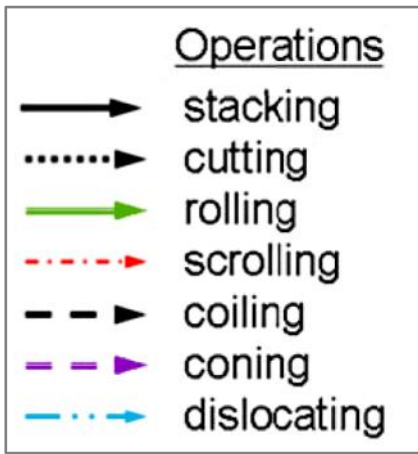
The “Grammar” of Fibrogenesis from Flat Materials

like C, GO, BN, MoS₂, WS₂, NbSe₂, ...

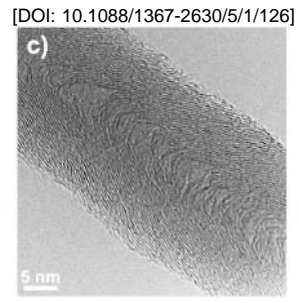


2. How big is the world of advanced fibres?

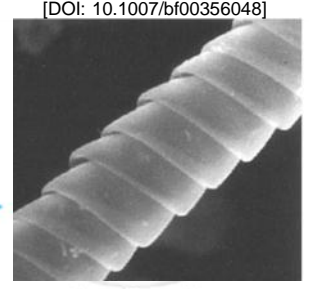
The **Products** of Fibrogenesis from Carbon



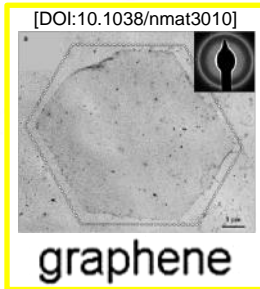
single-walled nanocone



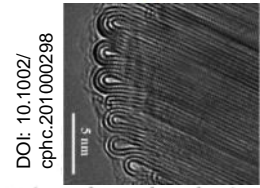
stacked-cone nanofibre



helical stacked-cone nanofibre



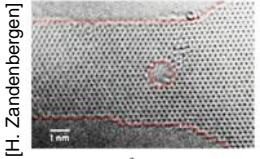
graphene



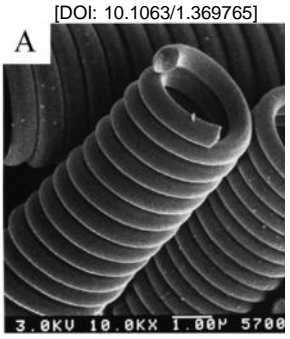
stacked-platelet nanofibre



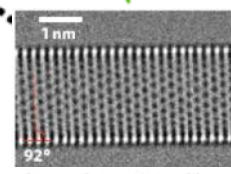
helical stacked-platelet nanofibre



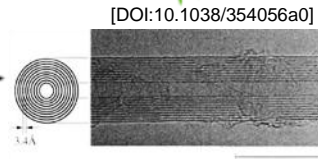
graphene ribbon



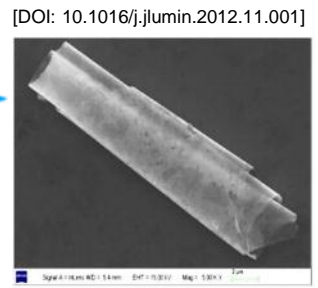
coiled nanotube



single-walled nanotube



multi-walled nanotube



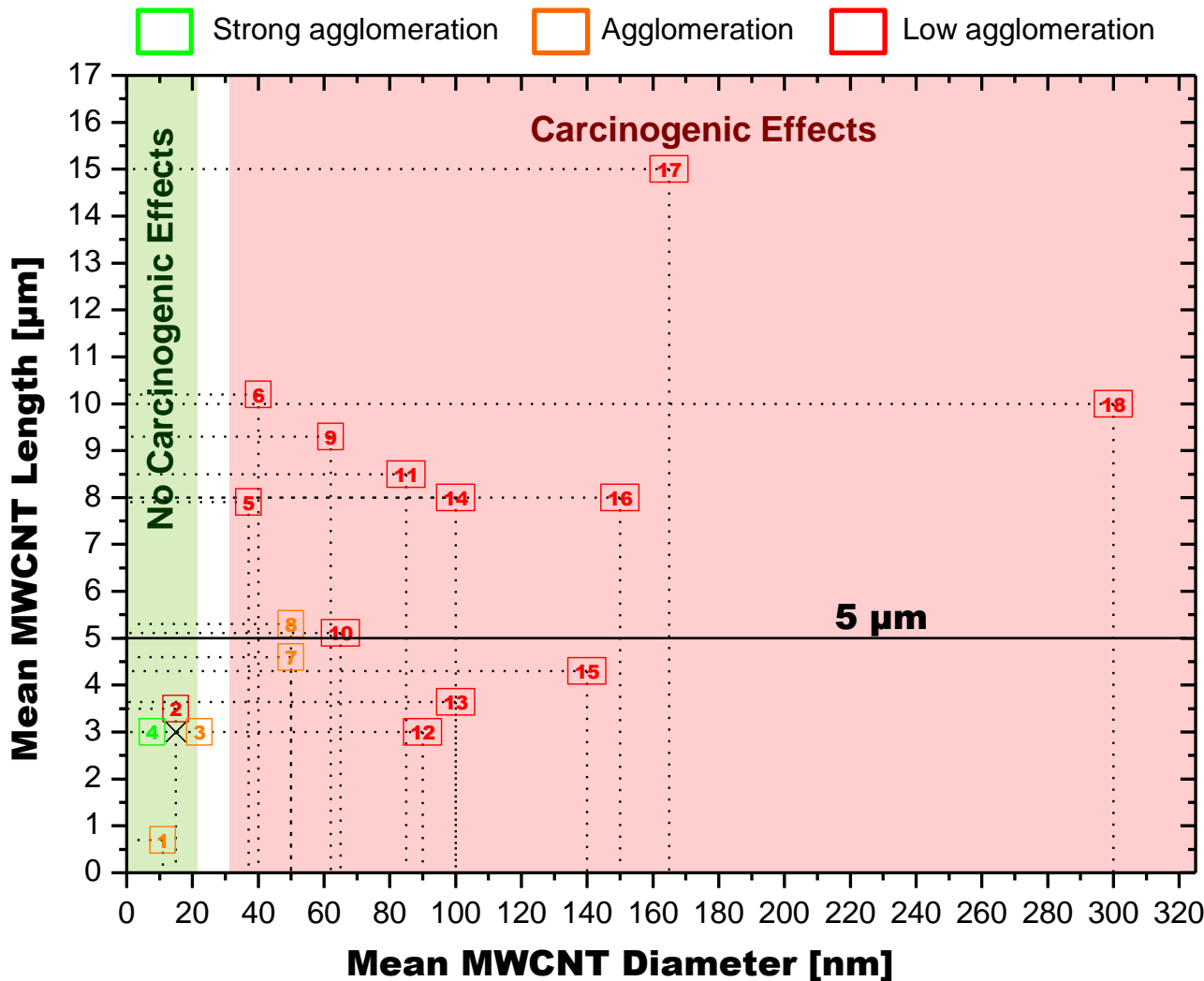
scrolled single-layer

... and many other

Materials: Nanowhiskers, nanowires, electrospun fibres, ...

3. Show biopersistent nanofibres fibre-like toxicity?

Literature on Carcinogenic Effects of MWCNTs during Intraperitoneal Testing



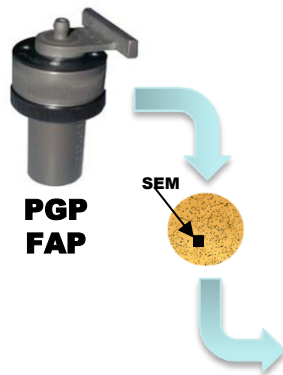
- [1] Muller, J., et al. (2009) Toxicol. Sci. 110(2): 442-448
- [2,17] Murphy, F. A., et al. (2011) Am. J. Pathol. 178(6): 2587-2600
- [3,16] Xu, J., et al. (2014) Cancer Sci. 105(7): 763-769
- [4,7-8,15] Nagai, H., et al. (2011) Proc. Natl. Acad. Sci. USA 108(49)
- [5-6,9,11] Rittinghausen, S., et al. (2014) Part. Fibre Toxicol. 11: 59
- [10,13] Xu, J., et al. (2012) Cancer Sci. 103(12): 2045-2050
- [12] Sakamoto, Y., et al. (2009) J. Toxicol. Sci. 34(1): 65-76
- [14] Takagi, A., et al. (2008) J. Toxicol. Sci. 33(1): 105-116
- [18] Sargent, L. M., et al. (2014) Part. Fibre Toxicol. 11: 3

[F. Herzberg, BfR]

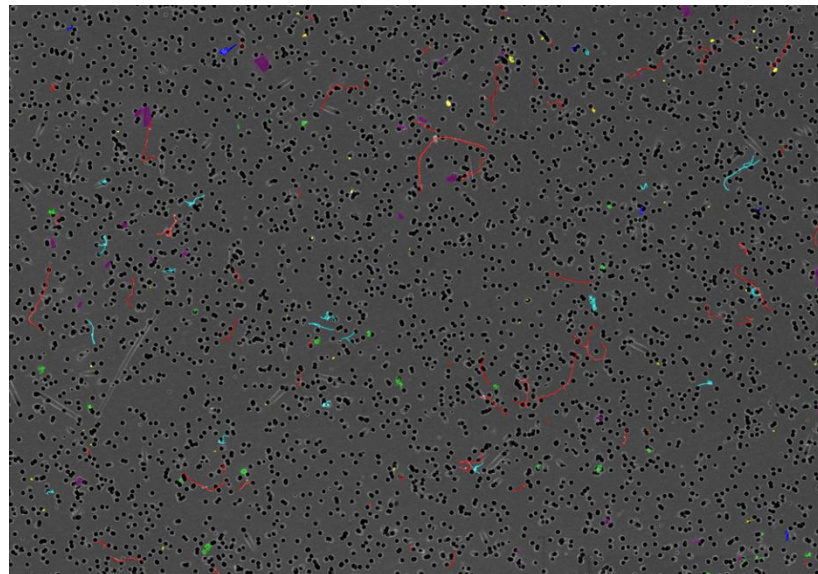
Yes ✓

Thus,

- Exposure levels in the order of $10\ 000\ \text{F}/\text{m}^3$ appear **justified** also for **nanofibres**
 - => REL in Announcement 527 (Manufactured Nanomaterials) 2013 of the German Committee on Hazardous Substances (AGS)
- Concentrations of $0.01\ \text{F}/\text{cm}^3$ are **not detectable** at WPs **by nanoparticle counters** like CPCs or **gravimetry**,
see [Poster S2-P7](#)
- Instead, **filter sampling**, **EM analysis** and **fibre counting** is currently the only way to assess nanofibre exposure



Individual Fiber (HARFO)
HAR Fiber Cluster (HARFC)
HAR Fibre Agglomerate (HARFA)
LAR Fiber Agglomerate (LARFA)
Particle Agglomerate (LARPA)
Individual Particle (LARPO)

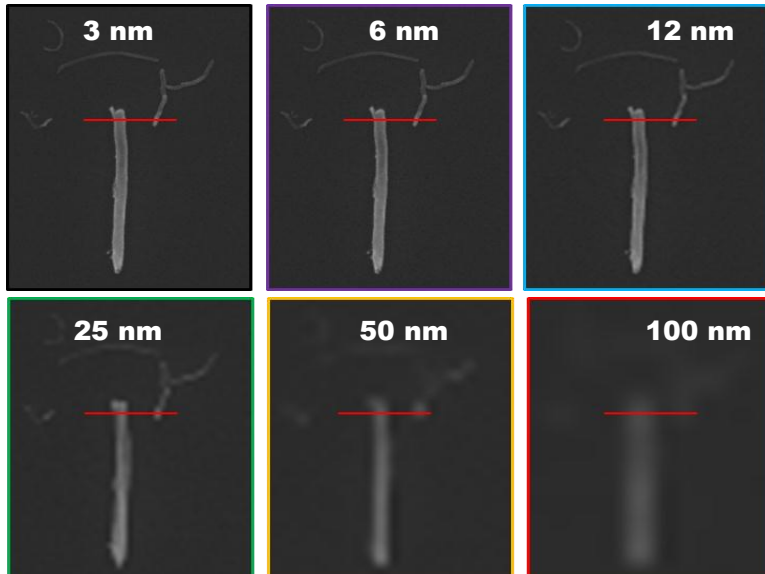


Classified SEM image of Arry ARIGM001 CNT aerosol
NDX150420_097_09_02_001_Low

... it needs guidance!

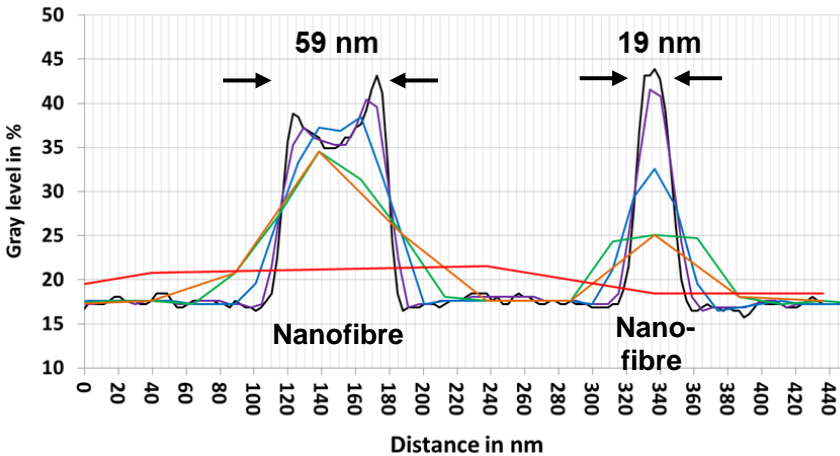
What EM Image Resolution is Necessary?

SEM images of varied resolution



Pixel Resolution

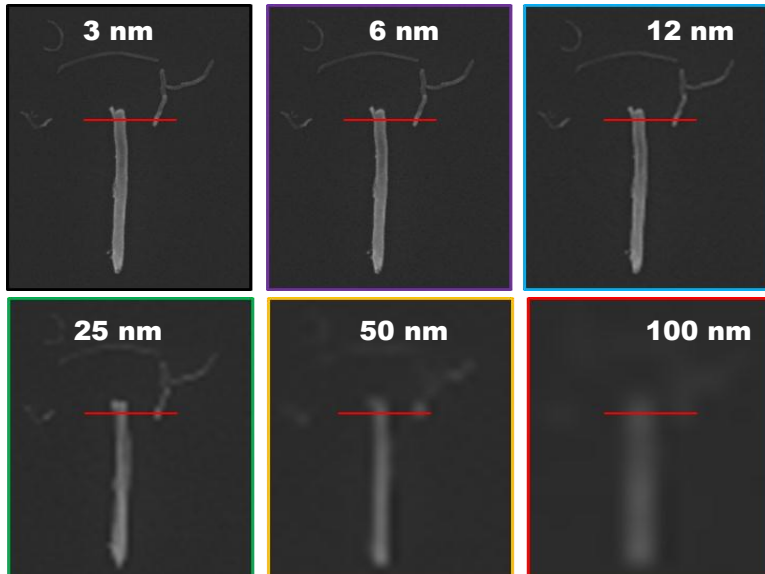
— 3 nm — 6 nm — 12 nm — 25 nm — 50 nm — 100 nm



Pixel resolution \leq Fibre diameter

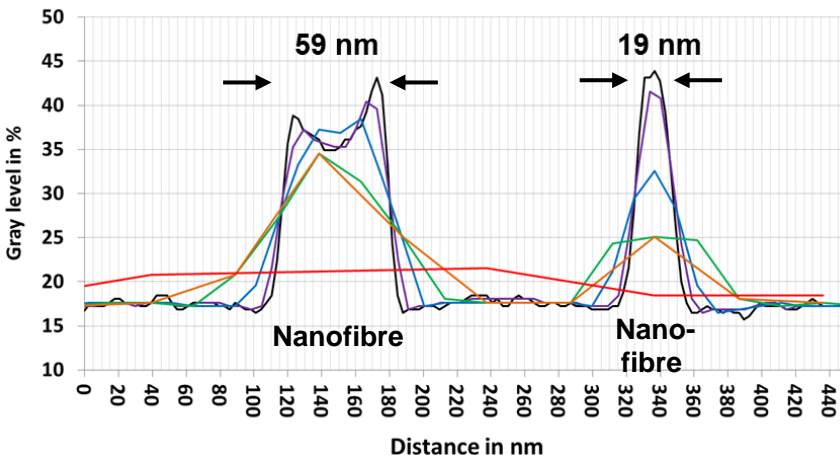
What Image Numbers are Necessary?

SEM images of varied resolution



Pixel Resolution

— 3 nm — 6 nm — 12 nm — 25 nm — 50 nm — 100 nm



Example:

Clearance Measurement

95% CL for less than **1000 Fibres/m³**
(sensitive to 333 fibres/m³)

3 litres room air per **0.5 mm² filter area**

VDI 3492

Flexible fibres to ignore

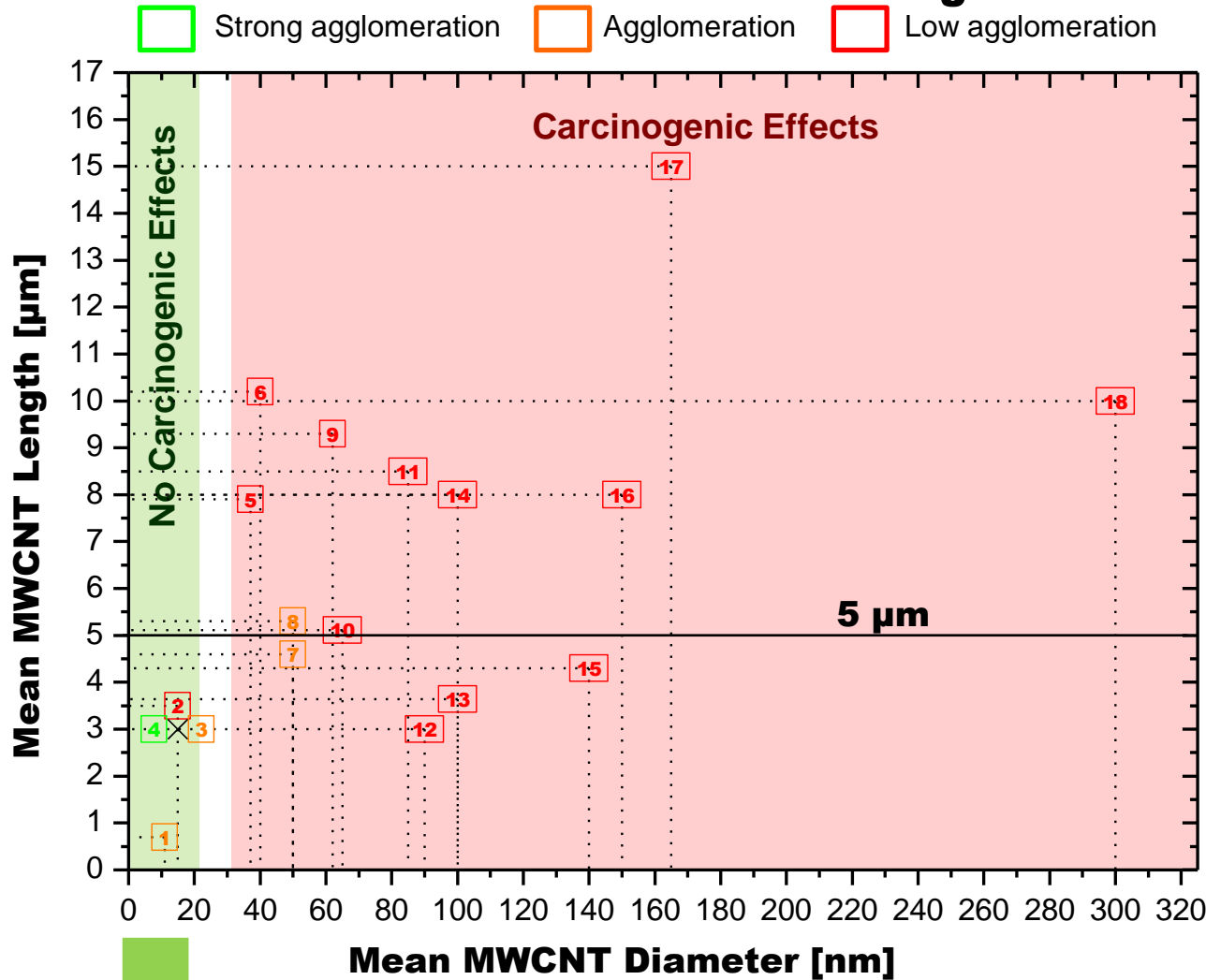
Rigid fibres to count

Image Pixel Resolution	Images of 1.2 MPixel to map 0.5 mm ²	Images of 20 MPixel to map 0.5 mm ²
1 nm	420 000	25 400
3 nm	46 700	2 825
6 nm	11 700	707
12 nm	2 920	177
25 nm	670	41
50 nm	168	10
100 nm	42	3

If we assessed **only THICK, RIGID nanofibres**, we reach at **manageable** numbers of **SEM images**

Justification to assess only thick nanofibres ...

Literature on Cancerogenic Effects of MWCNTs during Intraperitoneal Testing



[1] Muller, J., et al. (2009) Toxicol. Sci. 110(2): 442-448

[2,17] Murphy, F. A., et al. (2011) Am. J. Pathol. 178(6): 2587-2600

[3,16] Xu, J., et al. (2014) Cancer Sci. 105(7): 763-769

[4,7-8,15] Nagai, H., et al. (2011) Proc. Natl. Acad. Sci. USA 108(49)

[5-6,9,11] Rittinghausen, S., et al. (2014) Part. Fibre Toxicol. 11: 59

[10,13] Xu, J., et al. (2012) Cancer Sci. 103(12): 2045-2050

[12] Sakamoto, Y., et al. (2009) J. Toxicol. Sci. 34(1): 65-76

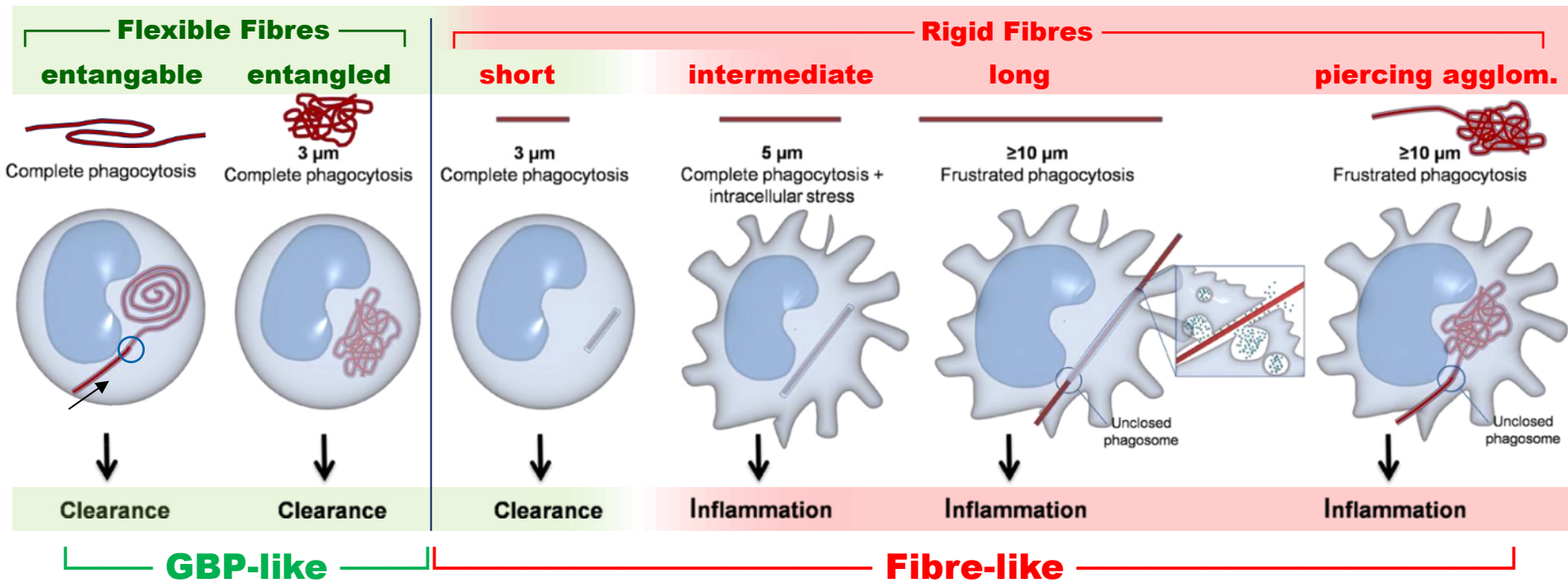
[14] Takagi, A., et al. (2008) J. Toxicol. Sci. 33(1): 105-116

[18] Sargent, L. M., et al. (2014) Part. Fibre Toxicol. 11: 3

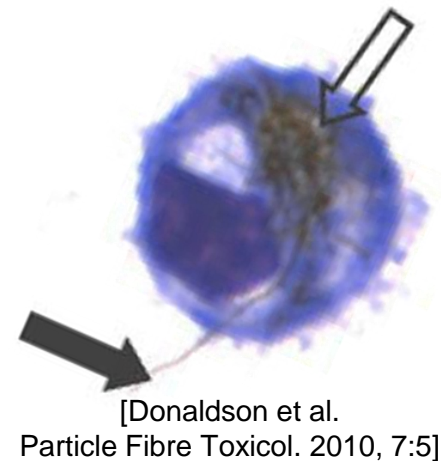
[F. Herzberg, BfR]

Hypothesis: Fibres thinner than 20-30 nm may behave not like fibrous but like granular biopersistent objects!

Complete vs. Frustrated Phagozytosis of **Entangable** vs. **Rigid** Fibres



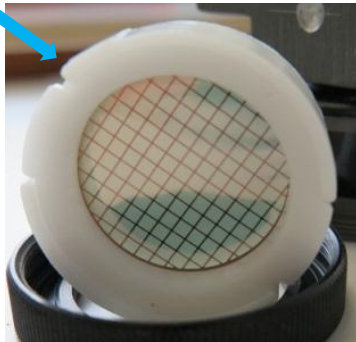
The **cells** and the **tissue** decide what level of **rigidity** is to be considered **critical!**



Practical Nanofibre Workplace Exposure Assessment

Filter Sampling

Nuclepore
200 nm Pore Size



Flow of 3 L/min for 2h 13min



SEM-analysis at **170** randomly-chosen filter positions

5120 x 3840 = 20 MPixels per SEM image

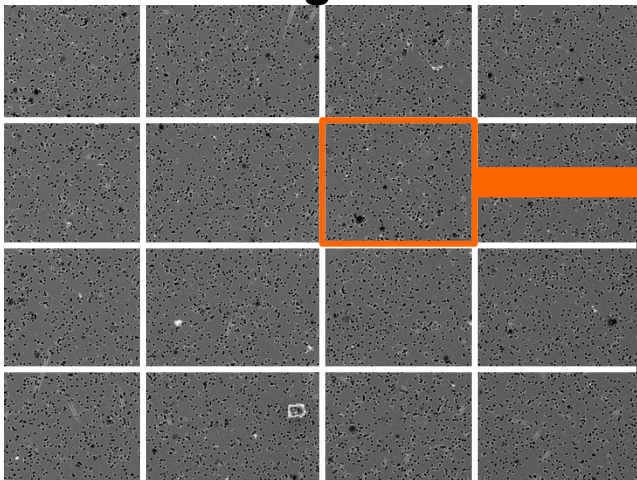
12.4 nm Pixel Resolution

0,5 mm² total filter area

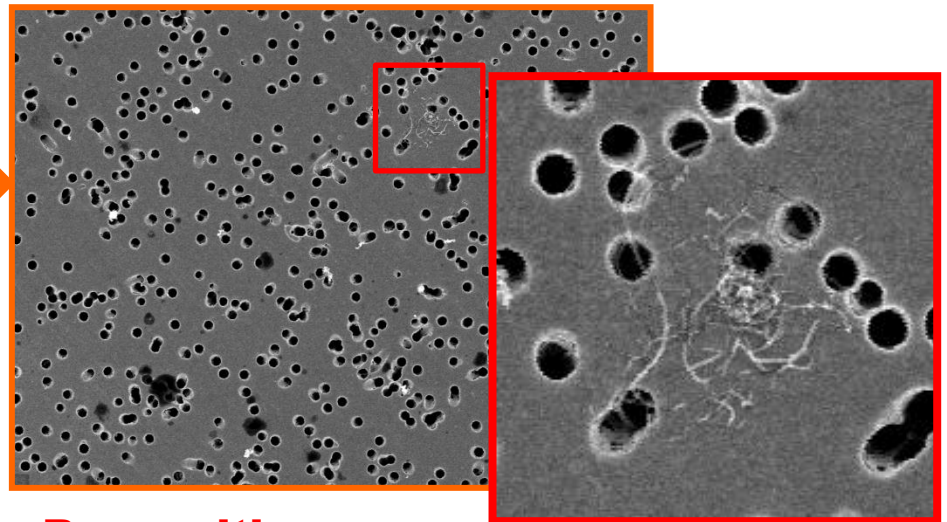
Detection Limit of **10 500 F/m³** at 95% CL

“Acceptance level” w/ RL 4·10⁻⁴

1 of 170 images



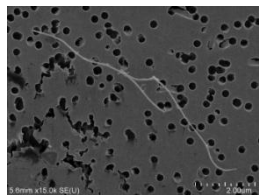
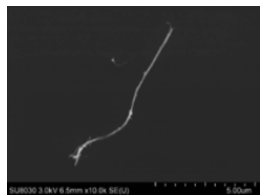
1 of 2720 sub images
for **pixel-wise visual inspection**



Recognition,
Classification and Counting of Objects

Suggested Categories for Counting

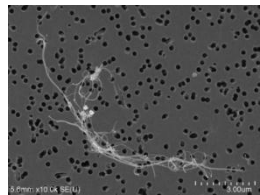
We propose to acquire **detailed** statistical information in four categories ...



**Long fibres $>5 \mu\text{m}$ &
Clustered long fibres**



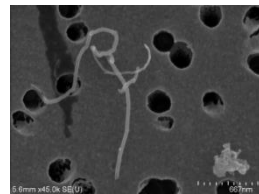
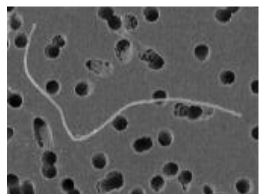
**individual count
to Cat. 1f (WHO)**



**Long
HAR agglomerates
 $>5 \mu\text{m}$ long AR 3:1**



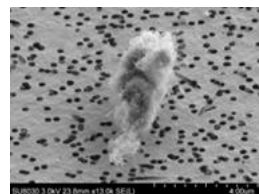
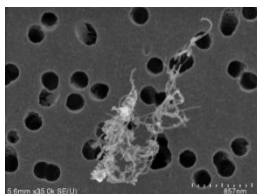
**single count
to Cat. 1a (WHO)**



**Short fibres $1-5 \mu\text{m}$ &
Clustered short fibres
(countable fibres)**



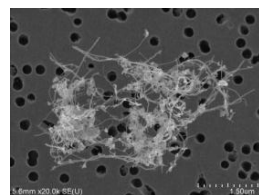
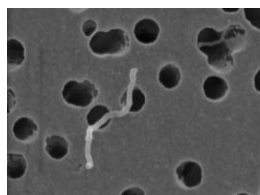
**individual count
to Cat. 2f**



**Short
HAR agglomerates
 $1-5 \mu\text{m}$ long AR 3:1**



**single count
to Cat. 2a**



**Fibres $< 1 \mu\text{m}$ &
LAR Agglomerates
(uncountable fibres)**



**! ignore !
Cat. 3a/f**

Countability of clustered fibres is the **biggest source of error** for dose assessment.

Detailed Counting Rules have been formulated

Zählregeln

- Als Faser im Sinne dieser Regel wird jedes Objekt gezählt, das eine Länge $L > 5 \mu\text{m}$, eine Breite $D < 3 \mu\text{m}$ und ein Länge/Breite-Verhältnis $L/D > 3 : 1$ aufweist. Als Länge gilt die rektifizierte Länge (L , siehe Abb. , als Breite die mittlere Breite. Es werden Fasern mit einem Durchmesser $D \geq 20 \text{ nm}$ gezählt (WHO-analoge CNT, **Kategorie III**). Fasern einer Länge zwischen 1 und $5 \mu\text{m}$ Länge werden gesondert gezählt (kurze CNT, **Kategorie I**). Fasern mit einer Länge $< 1 \mu\text{m}$ werden nicht gezählt (sehr kurze CNT, **Kategorie 0**), aber im Bericht erwähnt.

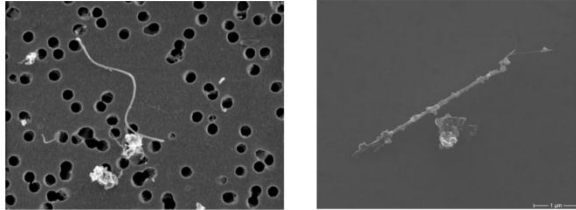


Abbildung 1: Einzelfasern (kurze Fasern nach Kategorie I – links, WHO Faser (Kategorie III – rechts).

- Bei Einzelfasern werden der Durchmesser (D) die Größe (G ; Durchmesser des umhüllenden Kreises) und die geschätzte rektifizierte Länge (L) angegeben.

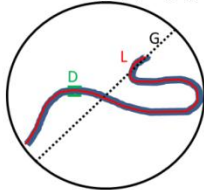


Abbildung 2: Durchmesser, rektifizierte Länge und Durchmesser des umhüllenden Kreises einer Faser.

- Ausbauchungen (wie sie beispielsweise durch Harz oder Binder bei künstlichen Mineralfasern auftreten können) werden ignoriert. Im Zweifelsfall wird $D < 3 \mu\text{m}$ angenommen (siehe **Abbildung 3**).
- Fasern, die an nicht faserförmige Partikel angelagert sind oder angelagert zu sein scheinen, werden behandelt, als ob die nicht faserförmigen Partikel nicht vorhanden wären. Es wird jedoch nur die sichtbare Länge der Fasern berücksichtigt, es sei denn, die Fasern gehen durch die Partikel hindurch und scheinen nicht unterbrochen zu sein.

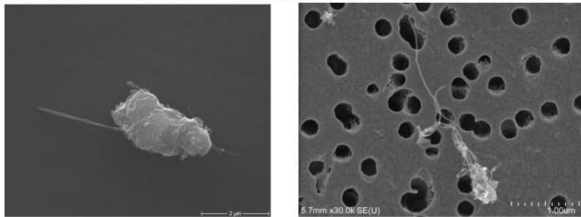


Abbildung 3: Fasern mit Ausbauchungen oder angelagerte Partikel.

- Ein Faserbündel, das an einer oder mehreren Stellen seiner Länge kompakt und ungeteilt erscheint, sich aber an anderen Stellen in separate Fasern zu teilen scheint (scheinbar aufgespleißte Faser), wird als 1 Faser angesehen. Ihre Breite wird in dem nicht

„aufgespleißten“ Teil gemessen. Jedes andere Agglomerat, in dem Fasern sich berühren oder kreuzen, wird als Faserbündel angesehen.

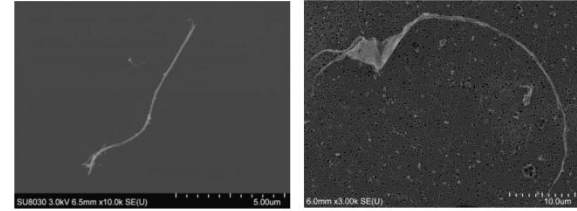


Abbildung 4: Faserbündel (aufgespleißte bzw. Faser mit faserförmigen Anhaftungen).

- Einander überlappende (überkreuzende) Fasern (Faserbündel) werden einzeln gezählt, wenn dies möglich ist (siehe Abb. 5).
- Überlappen sich so viele Fasern, dass sie nicht einzeln gezählt werden können (Faseragglomerat), so wird das Faseragglomerat nur dann als eine Faser gezählt, wenn seine Gesamtdimensionen die oben genannten Kriterien für Länge, Breite und Länge/Breite-Verhältnis erfüllen (faserförmiges Agglomerat). Andernfalls bleibt das Faseragglomerat unberücksichtigt (siehe Abb. 5).

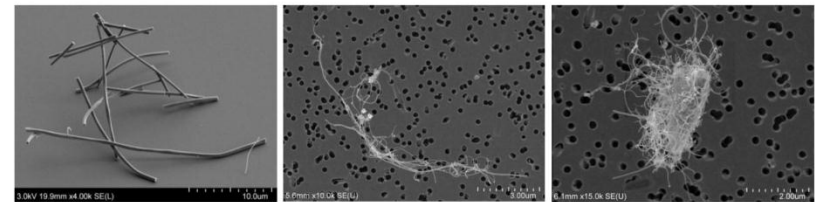


Abbildung 5: Faseragglomerat mit einzeln zu zählenden Fasern (nach Kategorie III) – links, faserförmiges Agglomerat (nach Kategorie IV) – Mitte und kurzes faserförmiges Agglomerat (Kategorie II) – rechts.

- Bei Faseragglomeraten, bei denen die Einzelfasern nicht mehr gezählt werden können, wird die Größe G und die Zahl der herausragenden CNT-Enden dokumentiert.

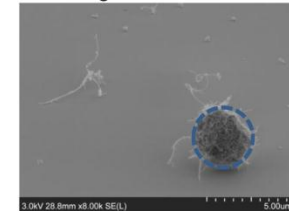
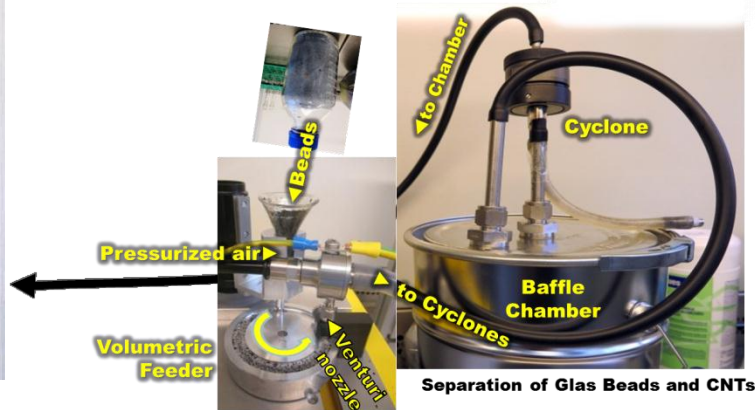
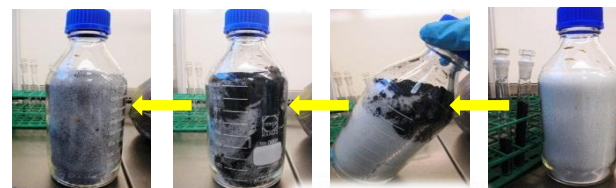
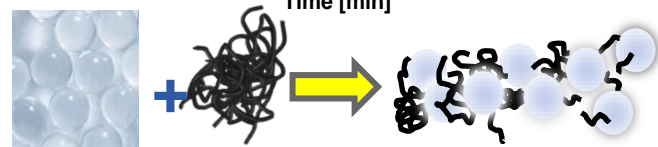
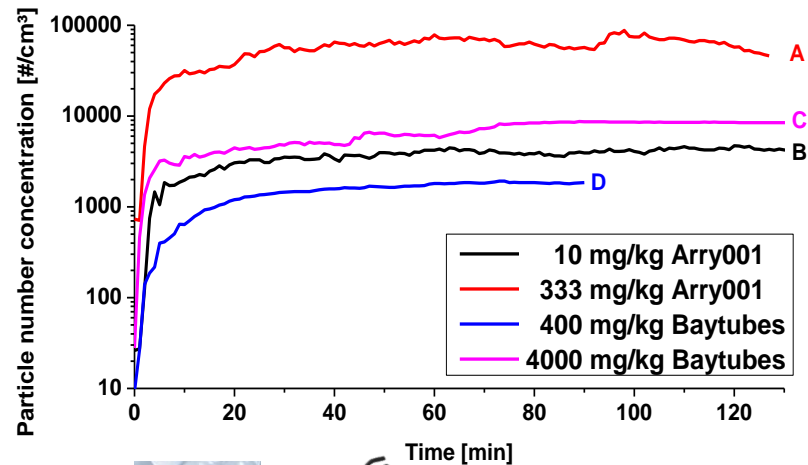
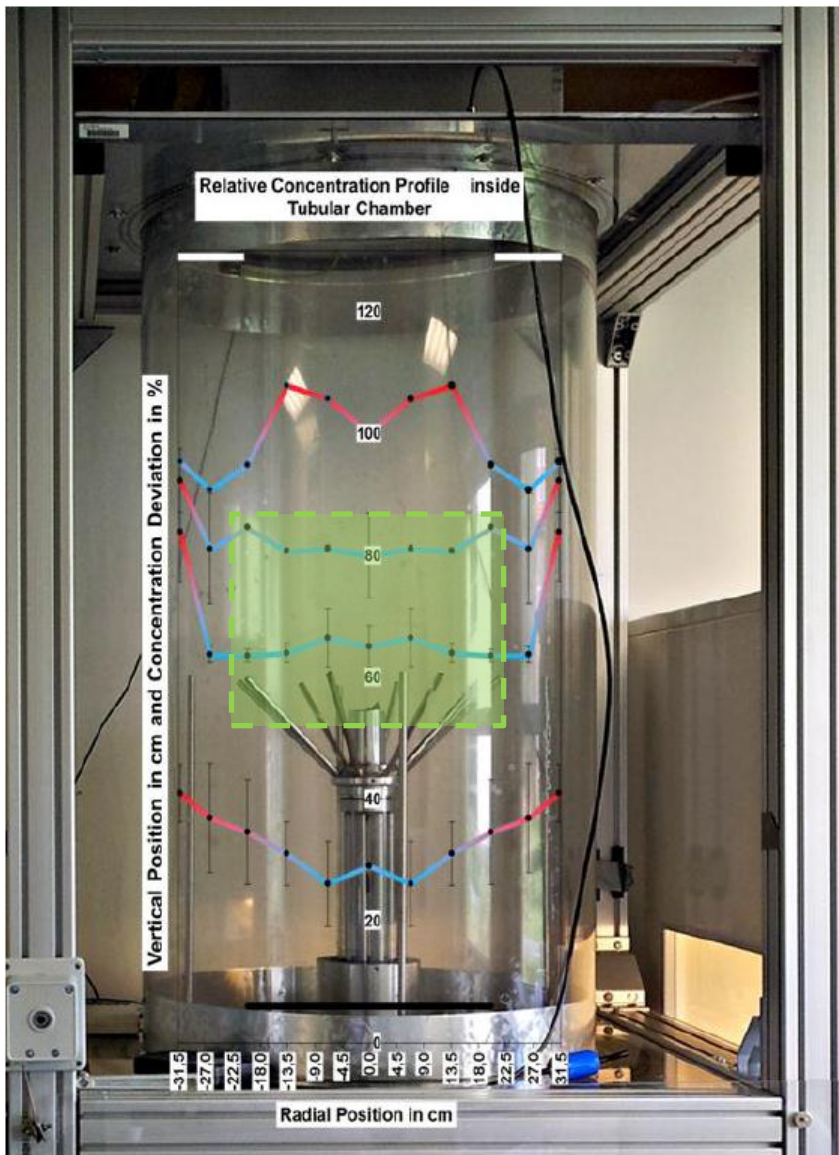


Abbildung 6: Faseragglomerat mit eingezeichneter Agglomeratgröße G .

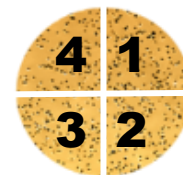
- Fasern, deren beide Enden im Inneren des Zählfeldes liegen, erhalten das Zählgewicht 1, Fasern mit nur einem Ende im Zählfeld erhalten das Zählgewicht $\frac{1}{2}$, Fasern mit beiden Enden außerhalb des Zählfeldes erhalten das Zählgewicht 0.
- Falls mehr als ein Achtel einer Zählfeldfläche von Fasern oder Partikeln bedeckt ist, wird dieses Zählfeld nicht berücksichtigt.
- Falls mehr als 10 % der Bildfelder einer Filterprobe als überbelegt gewertet werden, ist diese Filterprobe zu verwerfen.

Inter-Laboratory Nanofibre Counting Comparison at BAuA's 400-Litre Nanofibre Exposure Chamber

... see **Poster S2-P8**

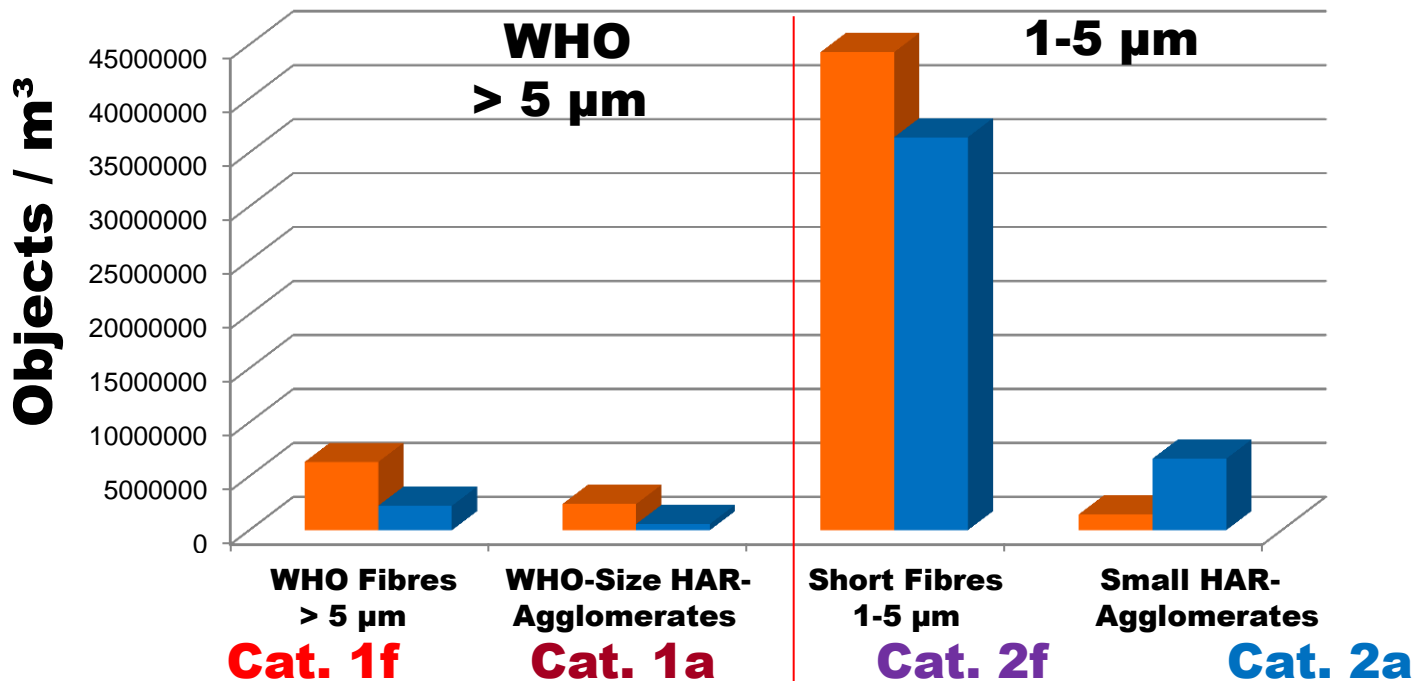


Inter-Laboratory Comparison w/ ARIGM001



1st Results on Visual Nanofibre Counting of SEM Images

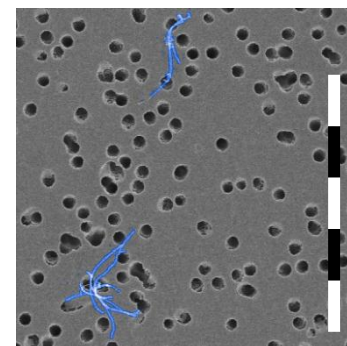
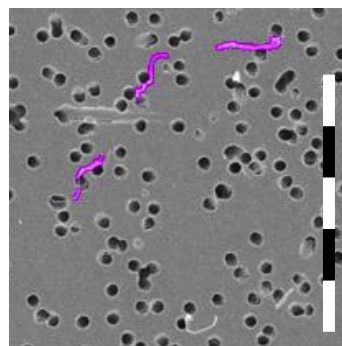
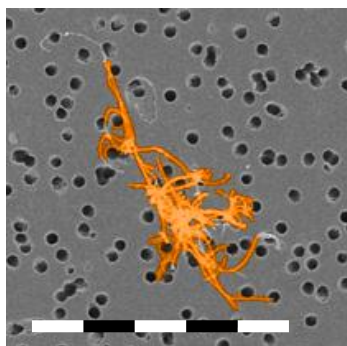
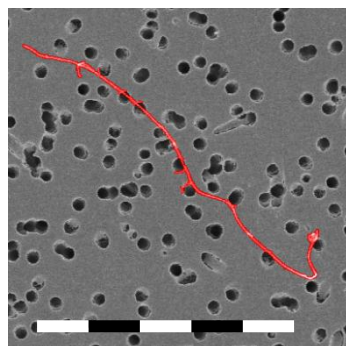
Sample: BAuA NGV 2016-01-25_004-06



■ **Partner 1**
 Σ 54M #/m³
 600 SEM images
 à 5 MPixel
 at 18.5 nm resol.

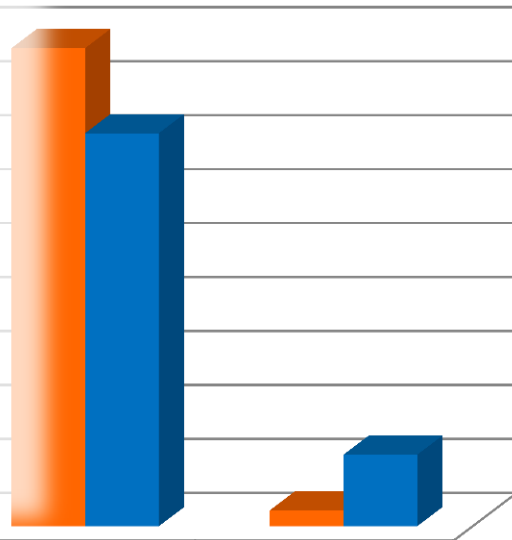
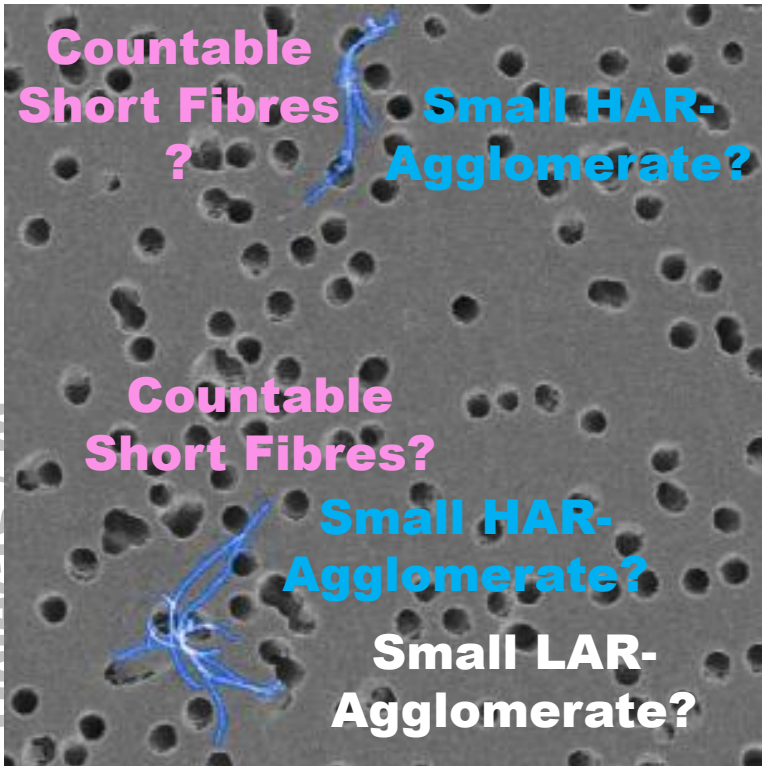
■ **Partner 2**
 Σ 46M #/m³
 50 SEM images
 à 20 MPixel
 at 16.5 nm resol.

■ **re-counting
& further
evaluations
still in
progress ...**



5 µm scale bar

Counting Ambiguities



■ **Partner 1**
 Σ 54M #/m³
 600 SEM images
 à 5 MPixel
 at 18.5 nm resol.

■ **Partner 2**
 Σ 46M #/m³
 50 SEM images
 à 20 MPixel
 at 16.5 nm resol.



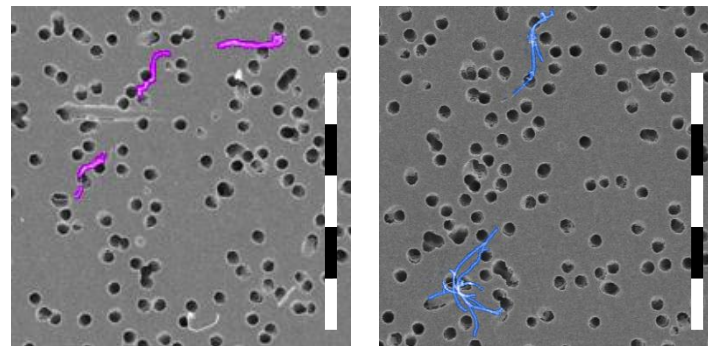
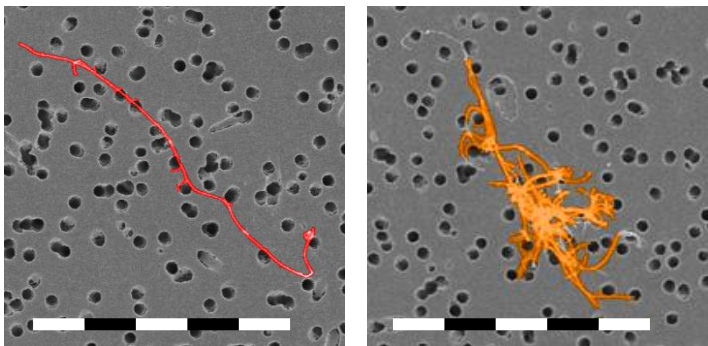
WHO Fibres > 5 μm
Cat. 1f

WHO-Size HAR-Agglomerates
Cat. 1a

Short Fibres 1-5 μm
Cat. 2f

Small HAR-Agglomerates
Cat. 2a

■ **re-counting & further evaluations**



5 μm scale bar

Cooperative Work

- Help to **improve** national and international WHO-fibre **sampling** and **counting guidelines** to make them **fit for the nanoworld!**
- If you **do fibre counting** and use EM, also count fibres thinner than **200 nm!**
- If you are interested to do **nanofibre counting**, we could provide sampled fibres and SEM images.
- Always specify **pixel resolution**, not **magnification!**
- Cooperate on **automatic** EM imaging and fibre counting!
- Let's **coherently assess** the world of **micro-** and **nanofibres** to **explore** the **prevalence** and **toxicological relevance** of H.A.R.M.!
(High Aspect Ratio Materials)

Respirability matters !

Automated Fibre Recognition

BAuA's Software FibreDetect

for fibre recognition, morphologic classification and counting
as well as fibre length, diameter and curvature statistics

FibreDetect 1.1.6 (Sep 27 2016) - fz.fib*

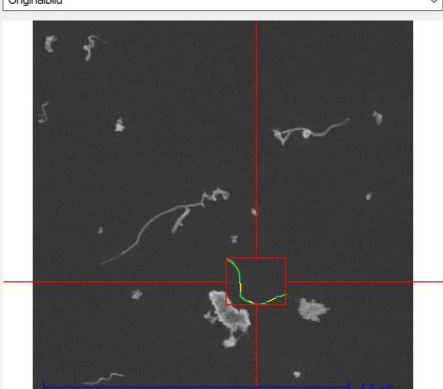
Datei Parameter

Bilderliste

S	Bildnr.	Ursprung
●	0001	f2_rgb.tif

Bildansicht

Originalbild



Objektklassifizierung

Kl	Typ	Status	Bild	Segment
●	F	automatisch	0001	0010
●	F	automatisch	0001	0003
●	A	automatisch	0001	0013
●	A	automatisch	0001	0006
●	A	automatisch	0001	0001
●	S	automatisch	0001	0017
●	S	automatisch	0001	0015
●	S	automatisch	0001	0014
●	S	automatisch	0001	0012
●	S	automatisch	0001	0011
●	S	automatisch	0001	0009
●	S	automatisch	0001	0008
●	S	automatisch	0001	0004
●	S	automatisch	0001	0002
●	U	keine	0001	0016
●	U	keine	0001	0007
●	U	keine	0001	0005

Ausschnitt aus dem Originalbild

Segment: 0010 aus Bild: 0001

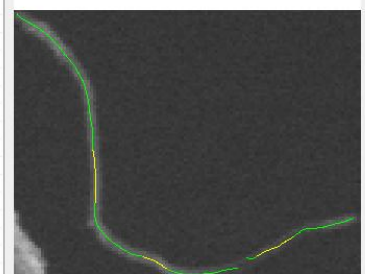
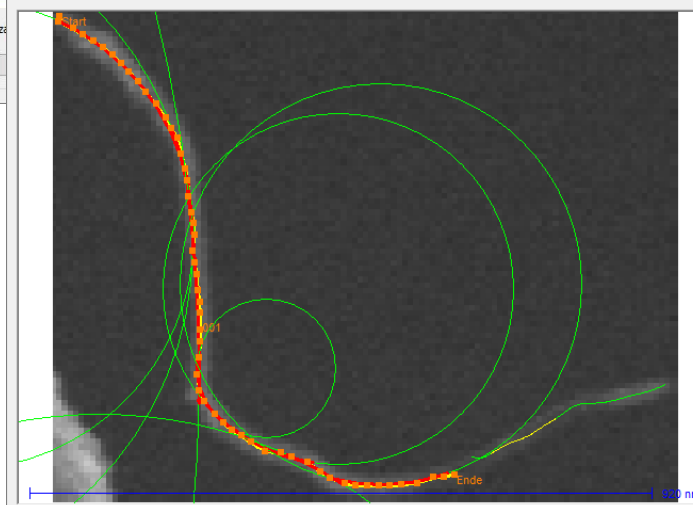


Bild anzeigen



Splineanz...

Spines

Bild
001
002

Kreise anzeigen

Name: 001, Länge [nm]: [1058.67], Winkelsummen links: [3.62] rechts: [2.27]

Automated Fibre Recognition

6 nm Pixelsize

12 nm Pixelsize

25 nm Pixelsize

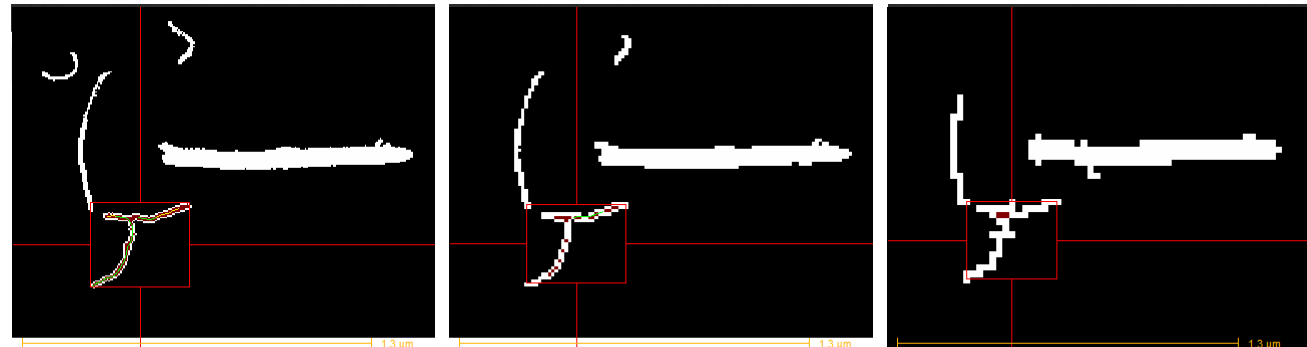
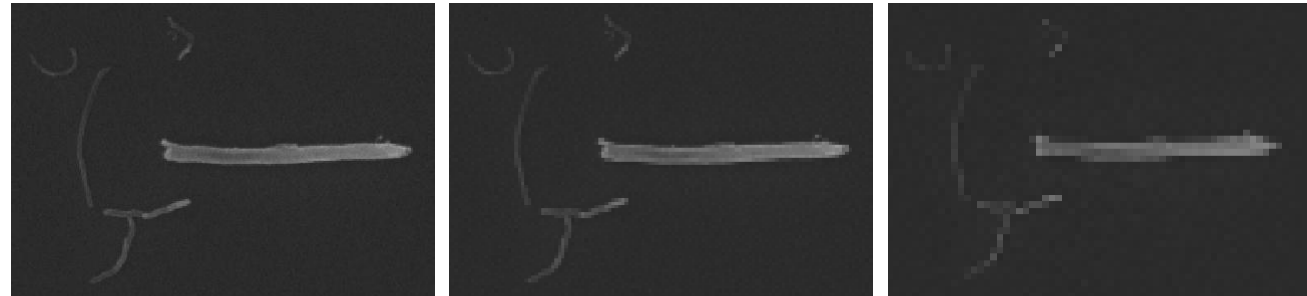
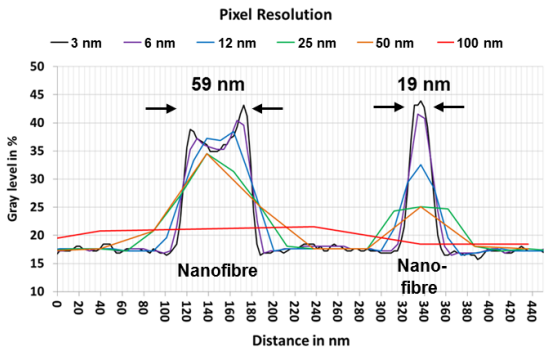
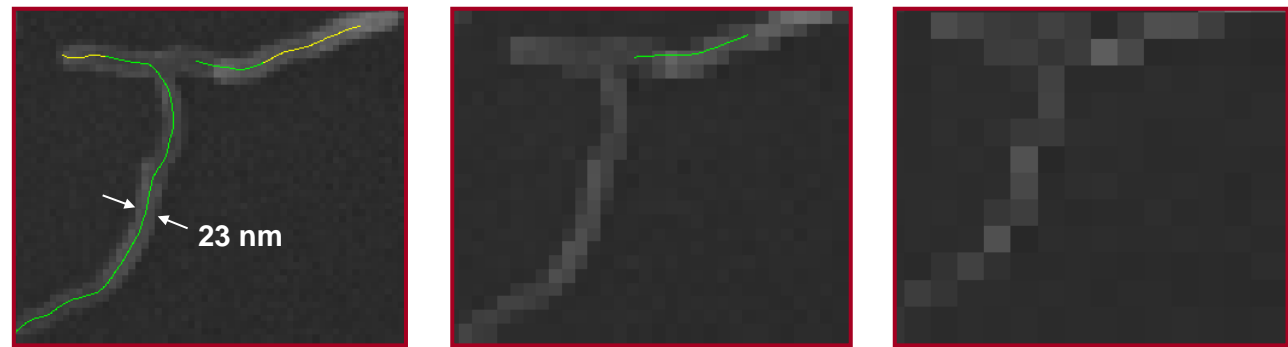
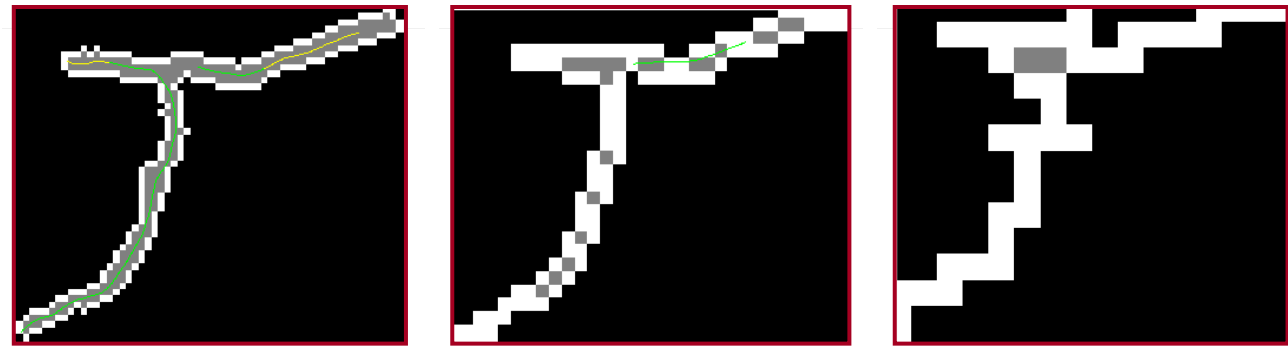


Image resolution and contrast are strongly related to fibre tracking issues



Thank you!