Image analysis:
How to convert micrographs to numbers

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The lecture was created for courses on Polymer Morphology.
Great majority of information in this lecture holds for non-polymeric materials as well.

Contents of this lecture

(1) IMA = IMage Analysis: explanation of basic terms
image processing = adjustment of B/C/G, cut outs, inserting scalebars…
image analysis = extracting quantitative information from micrographs

(2) Analysis of objects vs. analysis of fields:
analysis of objects (size and shape of a particle: EqDiameter, Circularity, Elongation…)
analysis of fields (structure coarseness, area and volume fractions, quantitative colors…)

(3) Examples of image analysis by means of ImageJ:
manual measurements: (i) calibration, (ii) interactive measurement, (iii) saving results
automatic measurements (i) segmentation (ii) object/field features, (iii) saving results
supplement: automatic segmentation → binary operations → manual adjustment

Intro & summary :: Outputs from EM and their processing

SEM and TEM outputs

1) Images = (real, direct) images = micrographs.

SEM/SE, SEM/BSE, SEM/STEM, SEM/EDX-maps
TEM/BF, TEM/DF, TEM/EDX-maps
...qualitative analysis and interpretation: lectures SEM+TEM
...quantitative analysis = **image analysis** = numeric outputs = this lecture

2) EDX spectra.

SEM/EDX, TEM/EDX
...qualitative analysis (which elements are present): lectures SEM+TEM
...quantitative analysis (how many of each element): software of your microscope

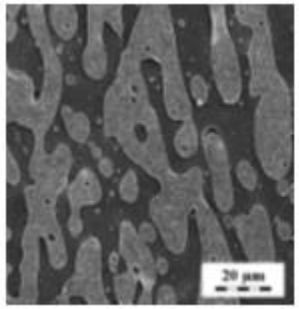
3) Diffractograms = diffraction images = diffraction patterns.

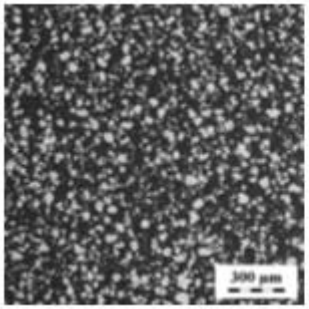
TEM/(SA)ED
...basics of diffraction theory and analysis: lectures TEM

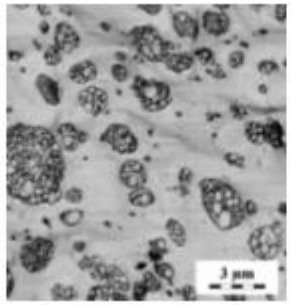
Image processing = adjustment of B/C/G, scalebars… → supplementary materials: ImageJ
Image analysis = extracting quantitative information from micrographs → this lecture

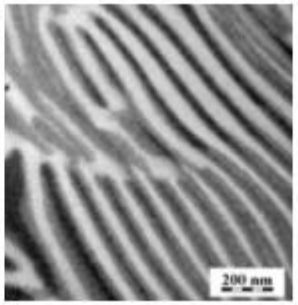
Why IMA = why to convert images to numbers?

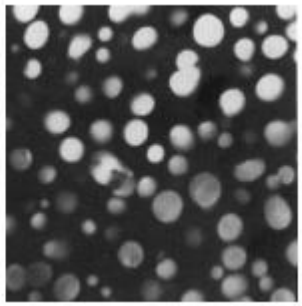
Calibration. Manual measurements. Segmentation & automatic measurements.

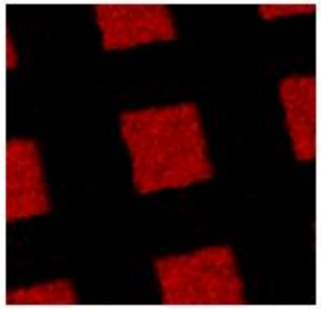

(1) SEM/SE


(2) SEM/BSE


(3) SEM/STEM


(4) TEM/BF


(5) TEM/DF


(6) EDXmap

Some questions that force us to convert images to numbers:

How big are the particles in images (2), (3) and (6)?
Which structure is finer (coarser) – the one in image (1) or (4) or (6)?
Which particles are more spherical – those on image (1) or (2) or (4) or (6)?
What is the area fraction and volume fraction of the particles/phases in all images?

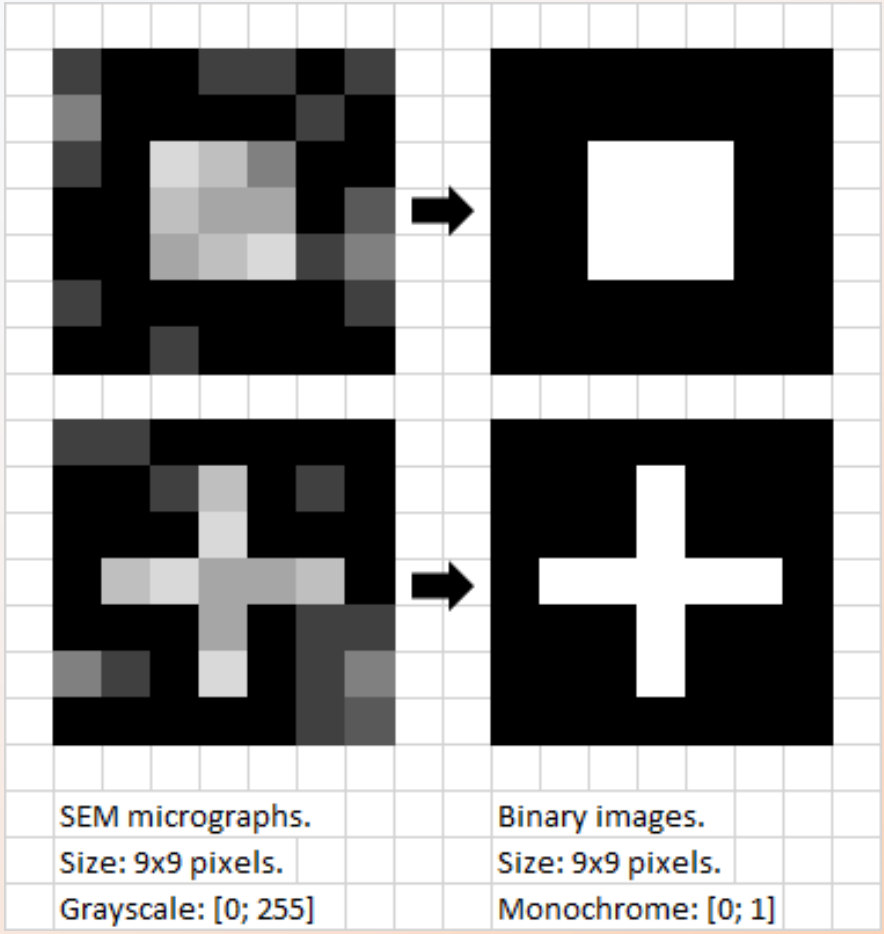
Calibration = determination of real width of image = pixel size
(ImageJ: measure a known length in an image… → see supplementary material: ImageJ

Manual measurements = interactive measurement of objects on the micrographs with a mouse
(ImageJ: process image, calibrate image, measure objects using your mouse… → [Ex.1]

Segmentation & automatic measurements
(segmentation = separation of objects from background to get binary image → [Ex.2]
(binary image = B&W image, where objects = white and background = black
(automatic measurement = software (NISe, ImageJ) extracts data from (binary) image

What is the size of (arbitrary) particle?

Answer: Image analysis → Object measurement → *EquivalentDiameter*



SEM micrographs.	Binary images.
Size: 9x9 pixels.	Size: 9x9 pixels.
Grayscale: [0; 255]	Monochrome: [0; 1]

Which is bigger, the square or the cross?

DEFINITION - EquivalentDiameter:
EqDia = $\sqrt{(4*\text{Area})/\pi}$

→ [Ex.3]

The square:
EqDia = $\sqrt{(4*9)/\pi}$ = 3.39μm

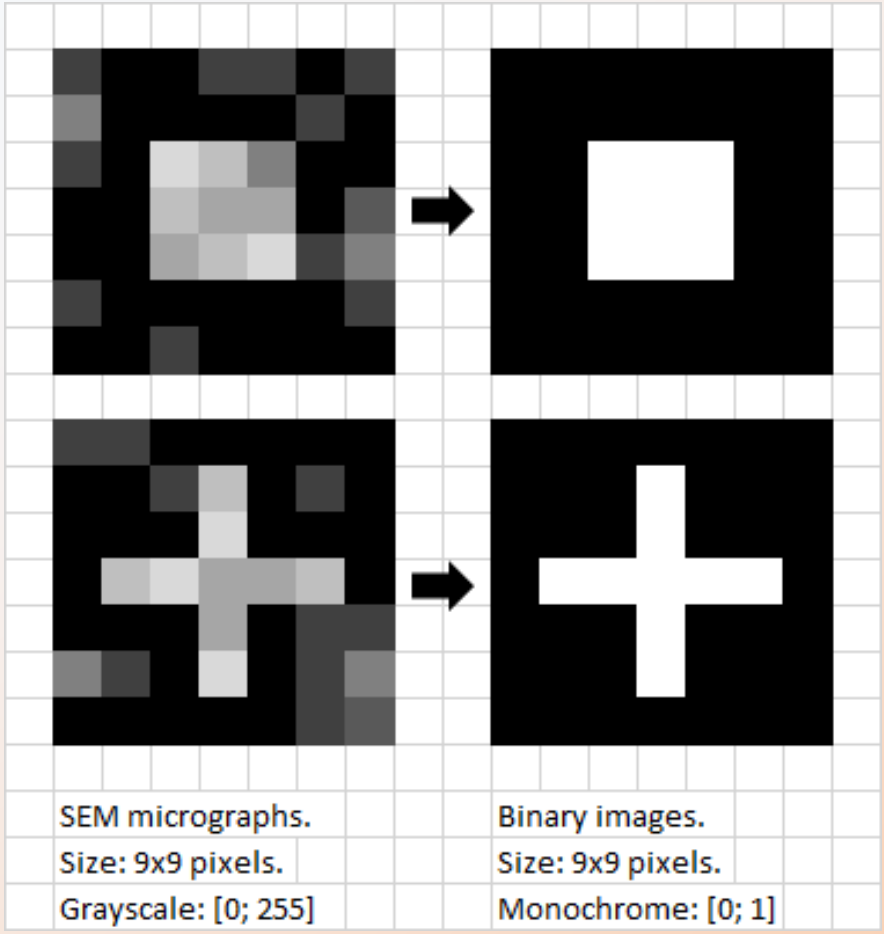
The cross:
EqDia = $\sqrt{(4*9)/\pi}$ = 3.39μm

Conclusion:
1) The two particles have the same size.
2) This works for arbitrary shapes.

From the point of view of IMA, micrographs are a 2D-array of pixels with values 0...255 = black...white.
Color images (each pixel has three values: red, green, and blue, intervals 0-255) – special case, see next.
Grayscale images (each pixel has one value within interval 0-255) – typical output from SEM and TEM.
Binary images (each pixel has is either black or white) – frequently used in IMA.
ImageJ works with all types of images; binary images are made using [Selections + ROI's + Masks].

Which particle is more spherical?

Answer: Image analysis → Object measurement → *Circularity, Elongation*



SEM micrographs.	Binary images.
Size: 9x9 pixels.	Size: 9x9 pixels.
Grayscale: [0; 255]	Monochrome: [0; 1]

Which is more spherical, the square or cross?

DEFINITIONS:

Circularity = $4\pi * (\text{Area} / \text{Perimeter}^2)$

Elongation = MaxFeret/MinFeret → [Ex.3]

Square:

Circ = $4\pi * 9/12^2 = 0.78$

Elong = $(3*\sqrt{2}) / 3 = 1.41$

Cross:

Circ = $4\pi * 9/20^2 = 0.53$

Elong = $5 / (3*\sqrt{2}) = 2.53$

Conclusions:

1) The cross is less spherical.
2) This works for arbitrary shapes.

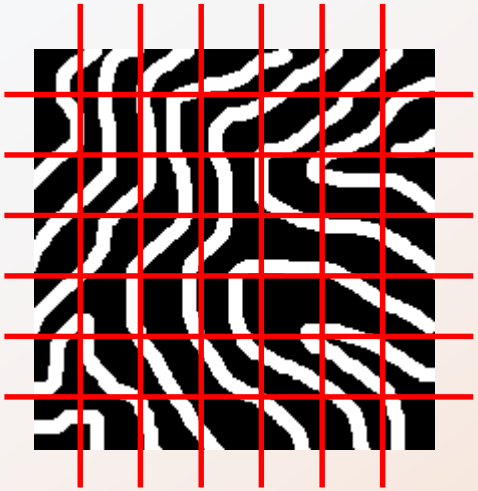
The key step in image analysis is usually the segmentation = conversion grayscale → binary image.

How do we make segmentation?

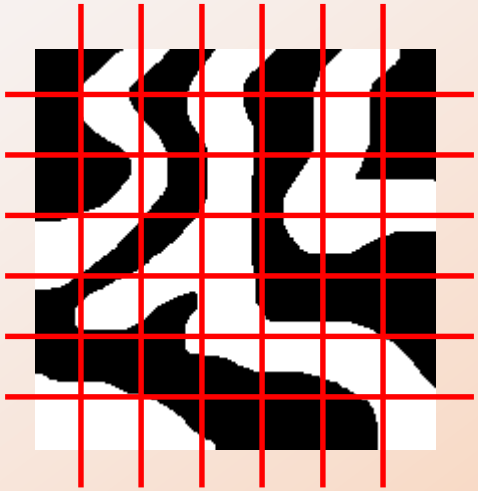
1) Theoretically: arbitrary image editor.
2) In real life: special software for image analysis, such as ImageJ.
3) Typical procedure: Threshold → Binary operations → Manual adjustment (see examples).

Which structure is finer?

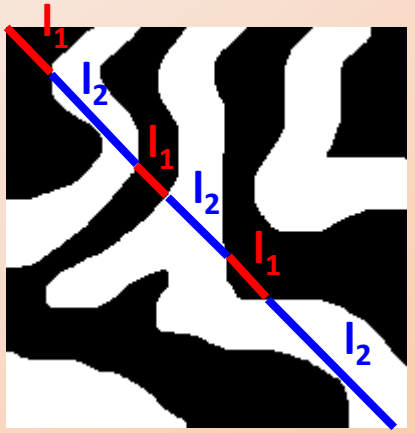
Answer: Image analysis → Field measurement → *MeanChord = MLI*



MeanChord = 1.2μm



MeanChord = 3.8μm



MeanChord = averaged length of lines l_1 and l_2.

Mean Chord = Mean Linear Intercept = MLI:

Definition: average length of line segments between two phases, averaged over all directions.

In IMA: average length of line segments ..... averaged over directions 0, 90 (45, 135) deg.

MeanChord can be determined also from SAXS (small-angle X-ray scattering).

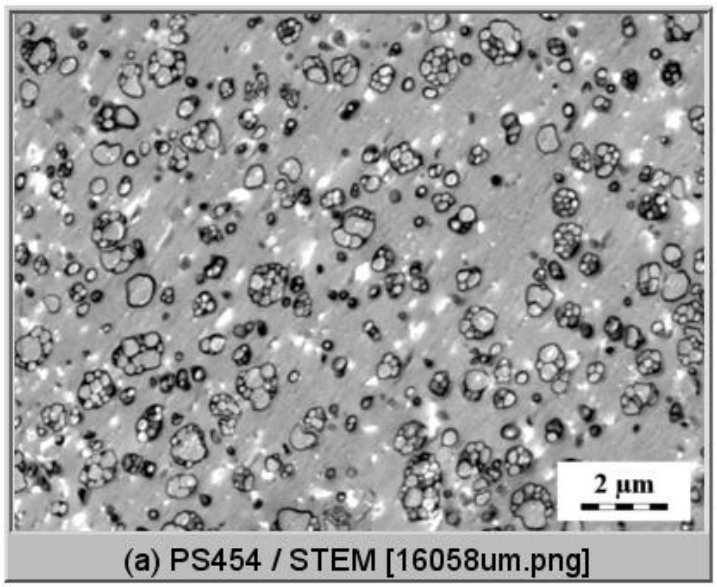
Principle: in SAXS, *MeanChord* is called *average chord length*. The SAXS calculation is based on Porod's Law, from which we get (for $qR_g >> 1$): $I(q)/Q \approx S/V*1/q^4$, $l_1 = 4\Phi_1 S/V$, $l_2 = 4\Phi_2 S/V$.

MeanChord is not in the standard ImageJ installation, but you can download a plugin:

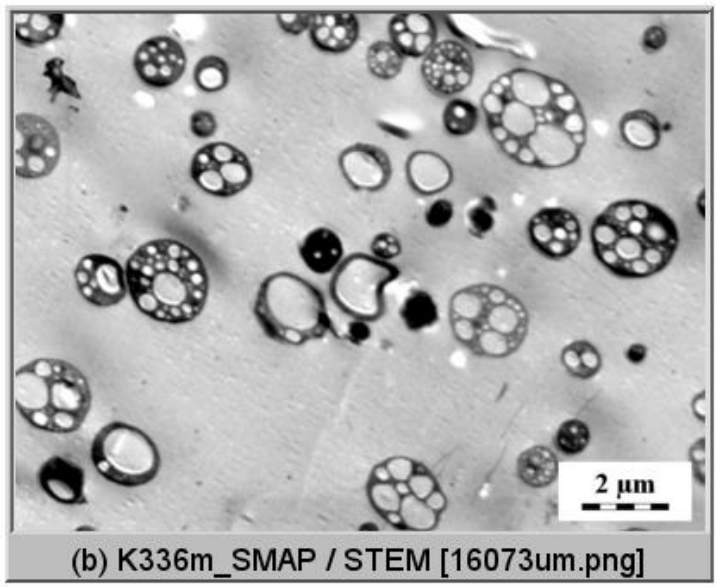
GoogleSearch: ImageJ mean linear inctercept ⇒ https://med.nyu.edu/nolanlab/software

What is the volume fraction of investigated phase?

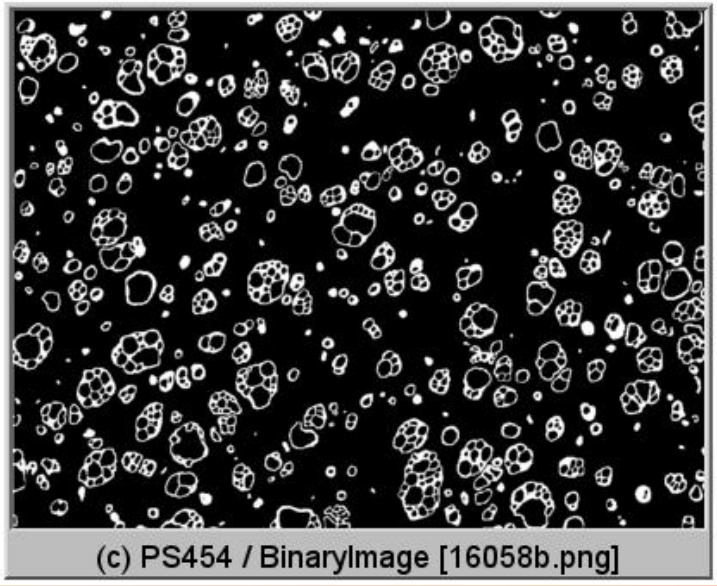
Answer: Image analysis → Field measurement → *AreaFraction…*



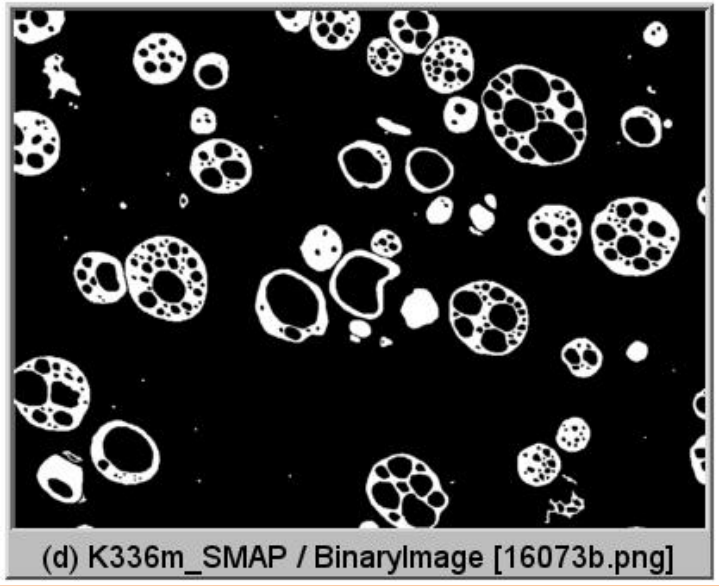
(a) PS454 / STEM [16058um.png]



(b) K336m_SMAP / STEM [16073um.png]



(c) PS454 / BinaryImage [16058b.png]



(d) K336m_SMAP / BinaryImage [16073b.png]

SEM/STEM micrograph of two HIPS polymers.

Question:
what is the volume of PB particles in PS matrix?

Answer:
surprisingly simple stereological formula

$$V_V \approx A_A$$

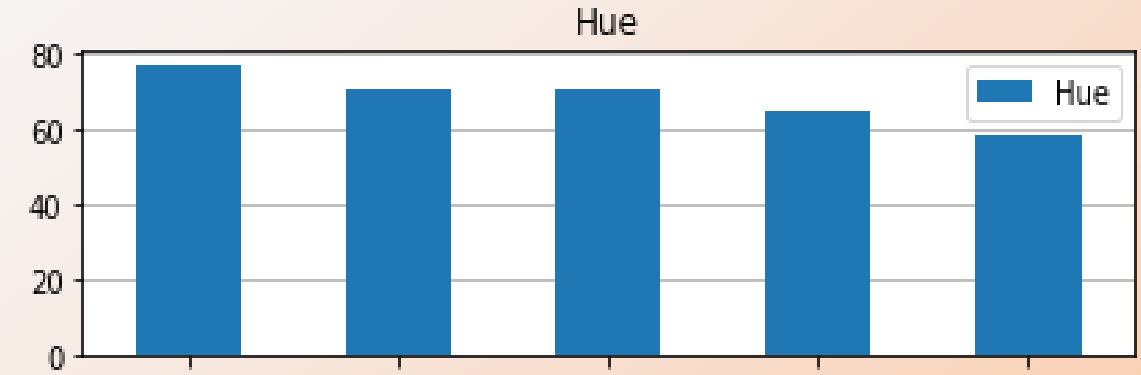
...volume fraction (volume in volume) is approximately equal to the area fraction (area in area).

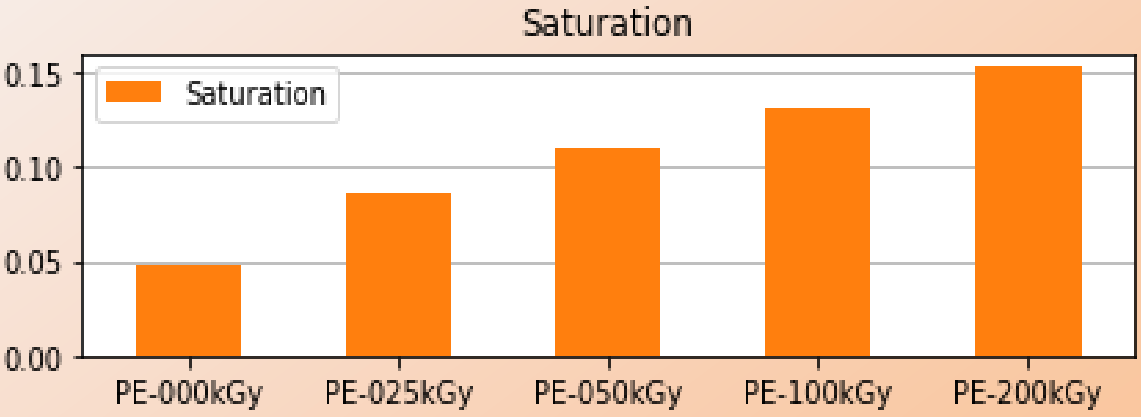
→ [Ex.4]

Can I detect (and quantify) color changes of my samples?

Answer: Image analysis → Field analysis → *MeanGray, RGB values, Hue, Saturation..*

↑ Light micrographs taken at the same illumination





A series of UHMWPE samples, which were irradiated by accelerated electrons in air.
According to IR, oxidative degradation increases with radiation dose.

Questions
Oxidation of UHMWPE means yellowing
⇒ can we see it?
⇒ can we prove it quantitatively by IMA?

Answers:
1) Naked eye: difficult.
2) IMA: clear and quantitative proof.

→ [Ex.5]

Conclusions & summary

- **The lecture was focused on IMage Analysis = IMA**
  = extracting of quantitative information from images and micrographs.

- **We showed to quantify the following features = morphological descriptors:**
  - *EqDiameter* = size of particle with arbitrary shape
  - *Circularity* and *Elongation* = numeric description of particle shapes
  - *MeanChord = MLI* = quantitative evaluation of structure fineness/coarseness
  - *AreaFraction* and *VolumeFraction* = area or volume occupied by investigated phase
  - Colors = quantitative evaluation of colors, by means of *Hue*, *Saturation* etc…

- **The image analyses can be performed with ImageJ program,**
  which has the following advantages:
  - Freeware – installation and usage is perfectly legal.
  - Well-established and widely used – lot of information in www.
  - Very popular among microscopists – there are even special ImageJ workshops.

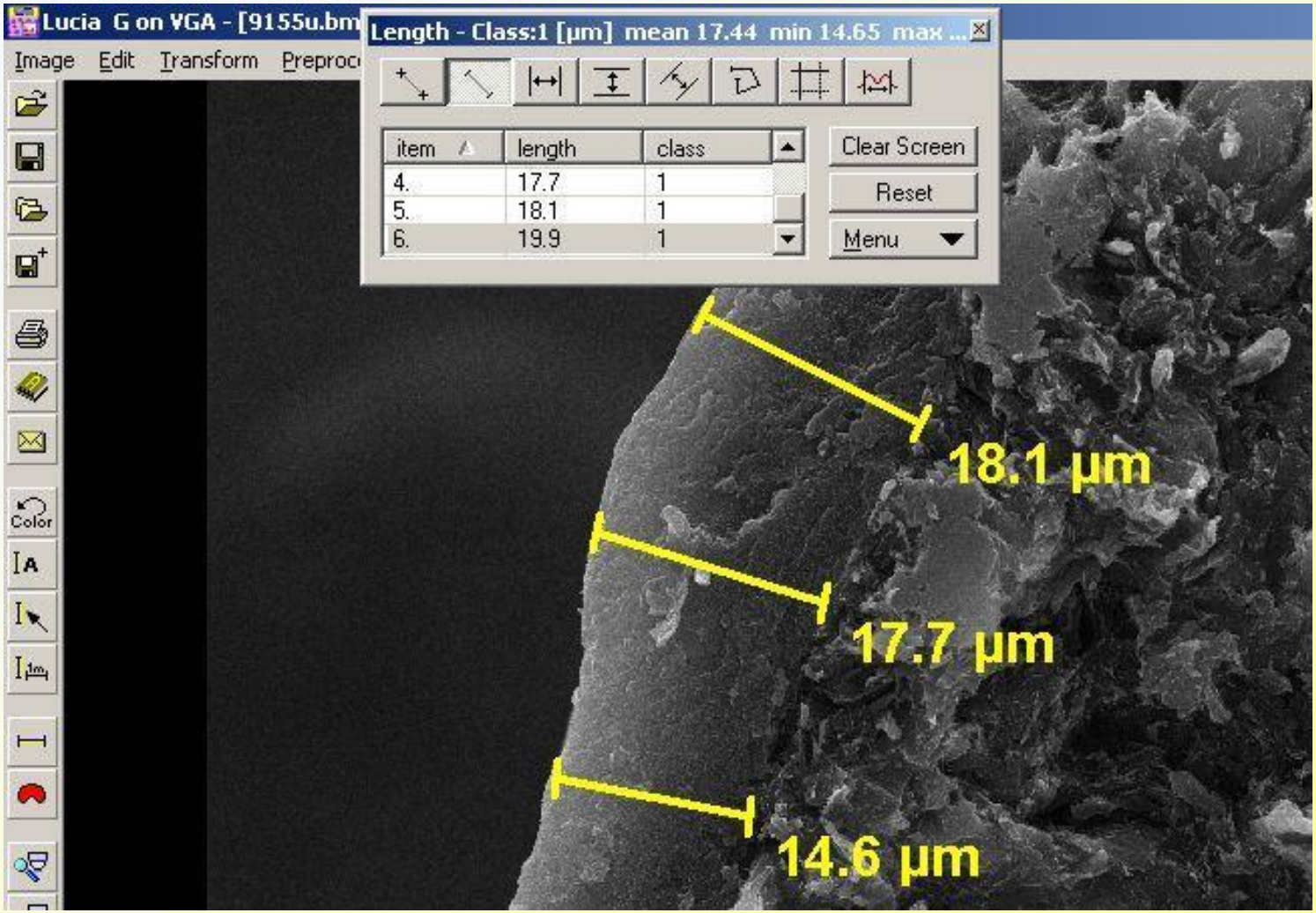
Image processing and analysis with ImageJ

- **Introduction to ImageJ** → supplementary material to introductory lecture EM0
- **Practical examples with ImageJ** → supplementary material to this lecture EM3

  Content of the subdirectory IMAGEJ (available on www-site):
  (https://mirekslouf.webnode.cz → Lectures for Trento → 3IMA → EXAMPLES
  - EX0 = basic processing of one micrograph + manual measurements
  - EX1 = processing of multiple micrographs + simple automation by IJM macros
  - EX2 = segmentation of simple image + automatic object measurement
  - EX3 = segmentation of more difficult image + automatic field measurement
- **Supplements** → the last slides of this presentation

  Comparison of morphological descriptors in ImageJ and NIS-elements
  Additional useful morphological descriptors
  References to case studies

[Example 1] IMA :: Manual measurements



SEM/SE micrograph of a pellet that is used for drug delivery.
The envelope of the pellet should be optimized for correct release of the drug contained inside.
Collaboration between IMC and Zentiva company.

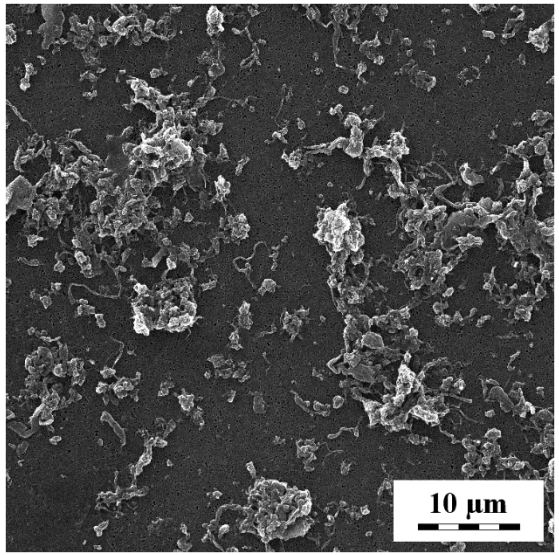
Calibration: we mark a known distance on the image and input information about its length.

Manual measurement: after calibration, we can measure lengths (areas, angles) with mouse...

Note: This example is in NIS-elements (formerly LUCIA) , but in ImageJ it works the same – see next.

[Example 2] IMA :: Automated measurements

Source data = SEM/SE micrograph of UHMWPE particles isolated on a polycarbonate membrane.

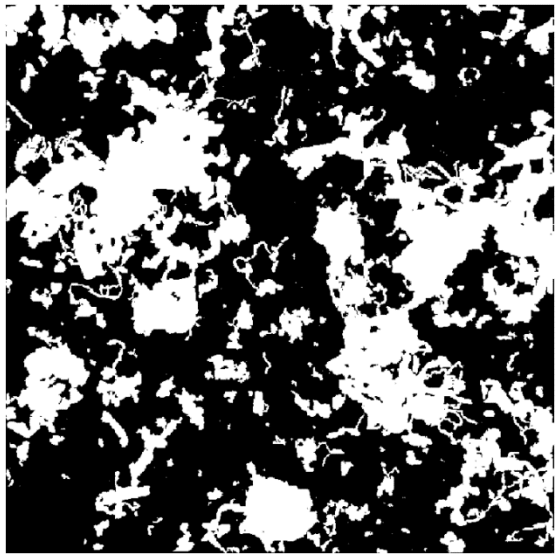



```
// [1] Ask user for input image.
_ImageOpen();

// [2] Open image, select what to measure.
MeasFrame(160,0,814,654);
ResetFieldFeatures();
SelectFieldFeature("AreaFraction");

// [3] Ask user to define/adjust threshold.
ViewBinary();
DefineThreshold(75,75,75,255,255,255,0);
_DefineThreshold();
Threshold();

// [4] Process image using binary operations.
CleanBinary(1,2);
DilateBinary(2,3);
CloseHolesBinary(3,3);
ErodeBinary(3,2);
CleanBinary(3,4);
SmoothBinary();
ViewOverlay();

// [5] Output the results.
MeasureField();
_FieldData();
```

Macro in program NISe, which converts the grayscale SEM/SE micrograph to binary image.

Result of IMA → the area covered by the particles is proportional to the volume of the particles.

```
=======================================
Program output:
=======================================

AF = AreaFraction

       Area_covered_by_particles
AF = ---------------------------
        Total_area_of_the_image


       White_areas_in_the_image
AF = ---------------------------
        Total_area_of_the_image

.......................................

Amount_of_wear_particles ~ AF

=======================================
AreaFraction (image 9081.bmp) = 36.62%
=======================================
```



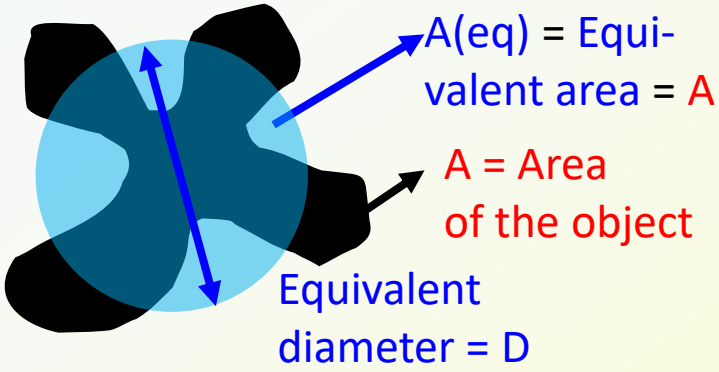
Binary image has just two colors:
white = objects
black = back-ground.

Source: M.Slouf et al. *Wear* 262 (2007) 1171-1181. (paper based on NISe; we will test this in ImageJ)

[Example 3] IMA :: Size and shape of arbitrary particles

Morphological descriptors – object features – size and shape of arbitrary particles

Particle size → EqDiameter



Computer measures directly:

A = area of the object/particle

We define:

A(eq) = area of a (hypothetical) circle with the area A

Therefore:

A(eq) = A

$A(eq) = 1/4 * \pi * D^2$

$D = (4A(eq)/\pi)^{1/2} = (4A/\pi)^{1/2}$

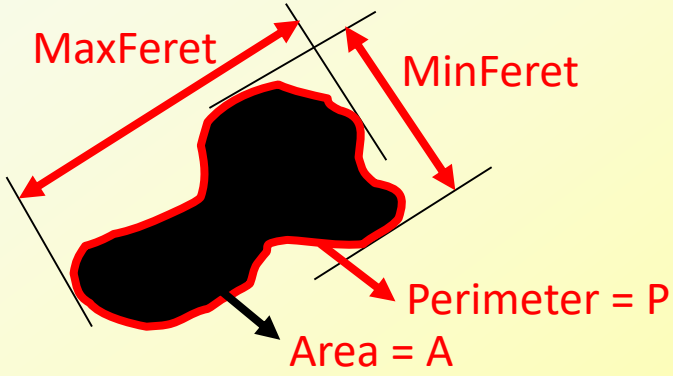
D = EqDiameter

Verbally:

EqDiameter is the diameter of a circle with the same area as the measured particle.

Particle shape

→ Circularity, Elongation...



Computer measures directly:

MaxFeret = maximal projection
MinFeret = minimal projection
Area = area of the particle
Perimeter = perimeter of the particle

We define:

C = Circularity $= 4 * \pi * Area / Perimeter^2$

E = Elongation = MaxFeret / MinFeret

Therefore:

for a circle ⇒ C = 1, E = 1

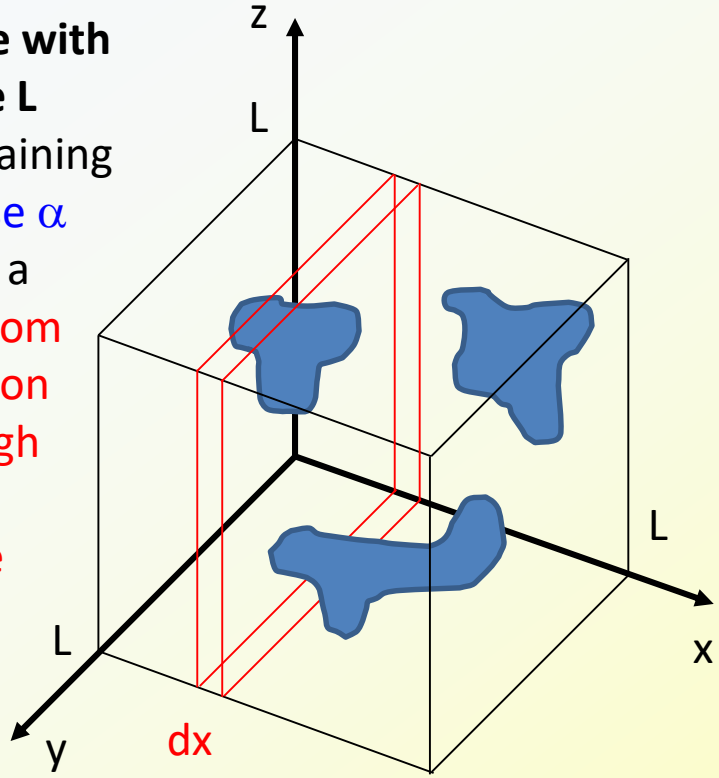
for a line ⇒ C = 0, E = ∞

Note:
red:
primary parameters determined directly by computer
blue:
derived parameters

Left as an exercise for the audience ☺

[Example 4] IMA :: Justification of $V_V \approx A_A$

Cube with edge L containing phase α with a random section trough the cube



$V_T = L^3$ = total volume of the cube

$V_V = V_\alpha / V_T$ = volume fraction of phase α (in cube)

V_α = total volume of phase α in the whole cube

$A_T = L^2$ = area of cut section

$A_A = A_\alpha / A_T$ = area fraction of phase α (in section)

A_α = total area of phase α in the whole section

$$dV_\alpha = A_\alpha(x)dx \quad \text{Eq.1}$$

$$\overline{A_\alpha} = 1/L \int_0^L A_\alpha(x)dx \quad \text{Eq.2}$$

$$V_\alpha = \int_0^L dV_\alpha = \int_0^L A_\alpha(x)dx = L\overline{A_\alpha} \quad \text{Eq.3}$$

Eq.(1): volume = base ∗ height

$A_\alpha(x) \rightarrow$ area fraction changes with *x*

Eq.(2): analogy with arithmetic mean

$\overline{A_\alpha} = 1/N \, \Sigma_i \, A_\alpha(i)$

Eq.(3): logic + substitutions from (1+2)

$V_\alpha = L\overline{A_\alpha}$

take Eq.3

divide by V_T

consider $LV_T = A_T$

$V_\alpha / V_T = L\overline{A_\alpha}/V_T = \overline{A_\alpha}/A_T$

$V_V = \overline{A_A} \approx A_A$

Q.E.D

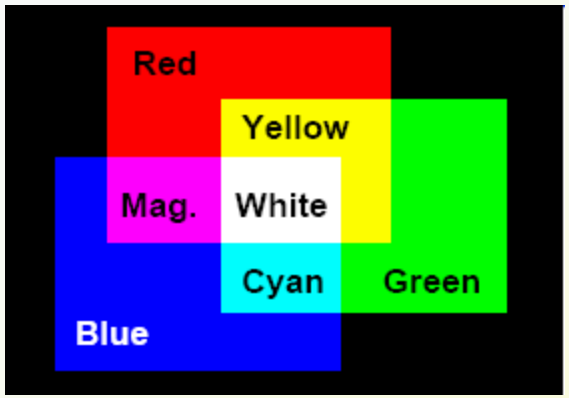
Key approximation: $A_A \approx \overline{A_A} \Rightarrow$ *AreaFraction* of α in a section = average *AreaFraction* of α.

[Example 5] IMA :: Quantification of colors – part 1

Color models, color spaces, conversion of colors to numbers.

RGB model

Additive color mixing



Black = no light = [R,G,B] = [0,0,0]
White = all pixels shine = [255,255,255]
Red = only red pixels = [R,G,B] = [255,0,0]
Green = only green pixels = [0,255,0]
Yellow = Red+Green [255,255,0] ...

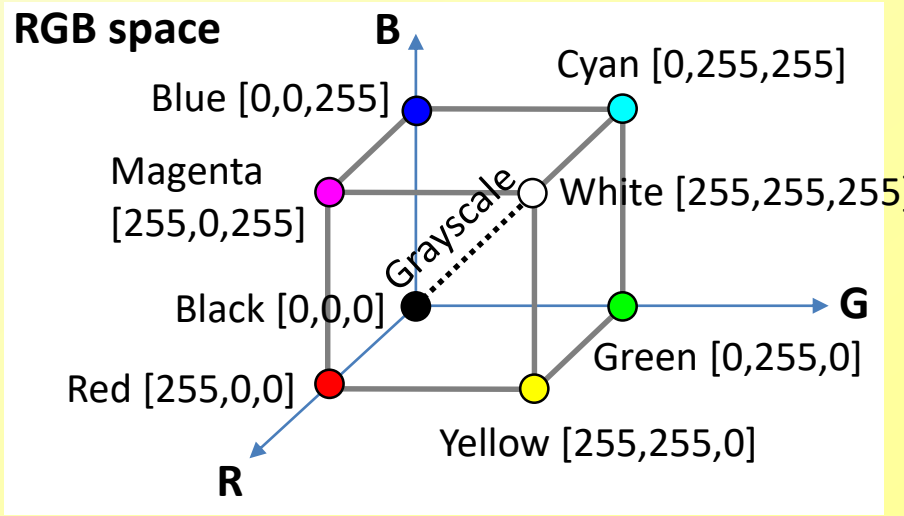
Summary:

1) Each color = 3 numbers = [R,G,B].
2) The three numbers can be regarded as coordinates, which gives RGB color space.

Alternative: HSV model

Less straightforward, more user-oriented.
As for colors, our eyes recognize namely:
1) Main color = Hue= H
   main wavelength, colors in circle: 0-360°
2) Intensity of the color = Saturation = S
   no color (grayscale) → intensive color: 0 → 1
3) Brightness of the color = Value = V
   no shine (black) → strong shine: 0 → 1

This gives us HSV color space (analogous to RGB).



Note: In computers we have: 1 byte = 2^8 bit = 256 bit ⇒ RGB values are (usually) in the range 0-255.

[Example 5] IMA :: Quantification of colors – part 2

Description of HSV model. Conversions RGB ↔ HSV in Jupyter/Python.

```
# Conversions between RGB and HSV color spaces in Jupyter/Python.
# (very easy: just import colorsys module from standard distribution
# (note: the numerical inputs/outputs in colorsys module are normalized to 1
```

```
import colorsys
```

```
# Blue (RGB -> HSV)
colorsys.rgb_to_hsv(0,0,1)
```

```
(0.6666666666666666, 1.0, 1)
```

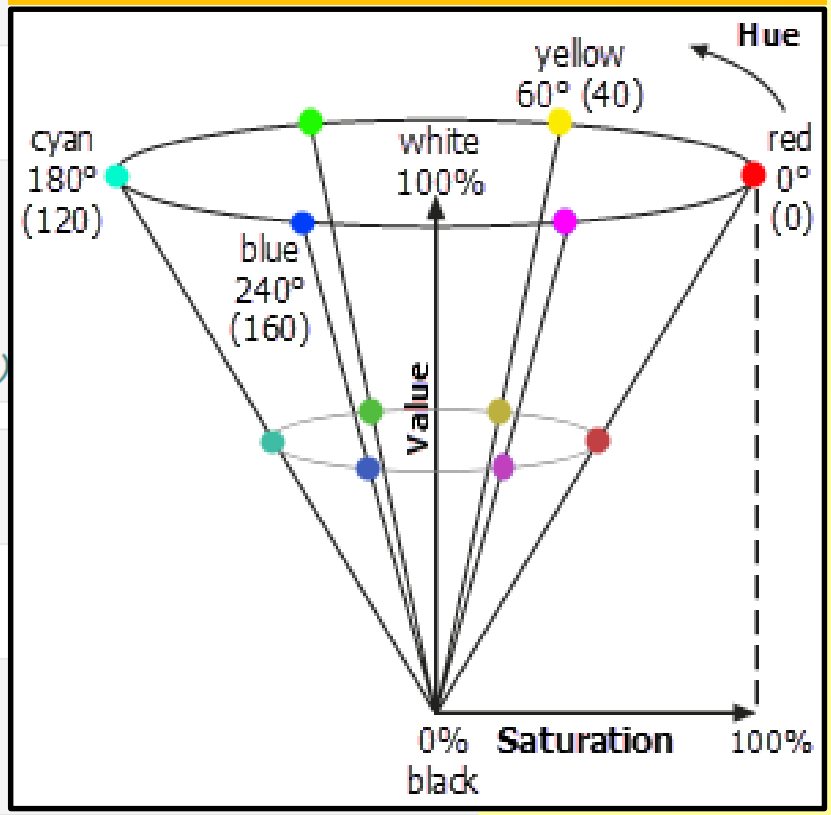
```
# Interpretation:
# * Hue = 2/3*360 = 240deg ~ blue color
#   (in HSV system: red,green,blue ~ 0,120,240deg
# * Saturation = clear color = saturation 1 (100%)
# * Value = color with maximum brightness = 1 (100%)
```

```
# Mean gray (RGB -> HSV)
colorsys.rgb_to_hsv(0.5,0.5,0.5)
```

```
(0.0, 0.0, 0.5)
```

```
# Interpretation:
# * Hue,Saturation = 0,0 => no color
# * Value = 0.5 ~ mean gray ~ intensity 0.5 (50%)
```

Description of HSV color space



Supplements

- Comparison of morphological descriptors in ImageJ and NIS-elements
- Additional useful morphological descriptors
- References to case studies

Supplement :: Morphological descriptors :: ImageJ vs. NIS elements

- ImageJ = free software with limited range of descriptors
- NIS-elements = commercial software with very broad range of descriptors

Two basic types of morphological descriptors:

→ for individual objects: object features – such as *EqDiameter*, *Circularity…*

→ for whole areas, micrographs: field features – such as *MeanChord, AreaFraction…*

The terminology and descriptors shown in the previous slides are from NIS elements.
Equivalent descriptors exist also in ImageJ, but the terminology is not so standardized.
(ImageJ, object features: Menu – Analyze – Analyze particles
(ImageJ, field features: Menu – Analyze – Analyze particles – Summarize

NIS elements	ImageJ
EqDiameter	Measure [*Area*] and recalculate: *EqDiameter* = $\sqrt{(4*Area/\pi)}$
Circularity	Measure [*Shape descriptors*], Circularity is among them
Elongation	Measure [*Feret's diameter*] and recalculate: *Elongation* = *Feret/MinFeret*
AreaFraction	Measure [*Area*] and use Menu – Analyze – Analyze particles – Summarize
MeanChord	Download a plugin: https://med.nyu.edu/nolanlab/software
Colors	Use pre-installed plugin: Menu – Plugins – Analyze – RGB measure

Conclusion: ImageJ is a well-established, high quality piece of free software, but concerning morphological descriptors it is not as elegant as the competing commercial NIS elements.

Supplement :: Additional useful morphological descriptors

- Again, the names of the descriptors come from NIS elements.
- Equivalents of these descriptors do exist (or can be calculated) also in ImageJ.

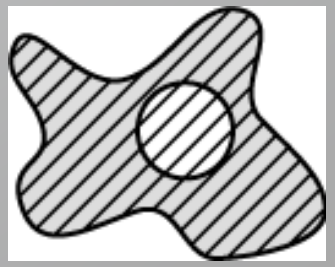
Area is a principal size criterion. In a non-calibrated system, it expresses the number of pixels; in a calibrated one, it expresses the real area. (object feature)

⇒ Area is a basic descriptor, it can be measured directly (number of pixels); it describes area/2d-projection of an object ≈ volume of object; main usage of Area consists in calculation of further descriptors (such as *EqDiameter*, *Circularity*, or *FillRatio* below)

AreaFraction is the ratio of the segmented image area and the MeasuredArea. It has a strong stereological interpretation: in the case of isotropic uniform random sections it is equal to the volume fraction. (field feature)

⇒ By means of AreaFraction we can estimate the volume fraction of the investigate phase; it is area of objects on the micrograph (A_A) and we suppose $A_A = V_V$ → [Ex.6]

FillArea**:** In case an object does not contain holes then the FillArea is equivalent to the Area. If an object contains holes, FillArea remains the same while Area is reduced by the area of the holes. (object feature)



FillRatio = *Area*/*FillArea*. (object feature)

⇒ the descriptor *FillRatio* differentiates objects with and without holes.

How in ImageJ? → combine previous slides + common sense + manual + www

Examples of image analysis in our publications

IMA is widely applied, because quantitative information is preferred in publications.

1) IMA employed in automated analysis of nanoparticles for multiple immunolabeling. We developed a script, which evaluates several morphological descriptors and differentiates larger Pd cubic nanoparticles from smaller spherical Au nanoparticles.

- M.Slouf et al. *Colloids and Surfaces B: Biointerfaces* 100 (2012) 205– 208

2) IMA employed in quantitative evaluation of yellowing of UHMWPE after e-beam crosslinking in air. Correlation between oxidation and color change was confirmed.

- M.Slouf et al. *J. Biomed. Mater. Res. Part B. Appl. Biomater*, 85B (2008) 240-251

3) IMA employed for quantification of UHMWPE wear particles. The isolated particles formed agglomerates which prevented direct counting. Our script yielded a good estimates of particle numbers based on the semi-automatic segmentation and their *AreaFraction*.

- M.Slouf et al. *Wear* 262 (2007) 1171-1181

4) Justification of the key stereological relationship $A_A = V_V$, i.e. area-in-area (area fraction some phase in the micrograph) = volume-in-volume (volume fraction of the phase).

- E.E.Underwood: *Quantitative Stereology*, pp. 25-27.

- ❖ The above publications (1-3) come from our laboratory, many other examples are in www.
- ❖ The publications are available on WebOfScience or at request (slouf@imc.cas.cz).