Lecture 2 |

Image Processing for Microscopy

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based on lecture notes from Prof. Vaso Tileli

Today we will cover:

- Correcting defects (e.g. uneven illumination)
- Image enhancement (improving visualization using filters, kernels etc.)

  histogram equalization, GAMMA value, kernel-filters
- Processing images in the frequency domain (FFT)
- Segmenting and thresholding
- Image measurements

Correcting Defects

Image processing operations and procedures that can be applied to correct some of the defects or shortcomings in as- acquired images that may occur due to imperfect detectors, limitations of the optics, inadequate or nonuniform illumination, or an undesirable viewpoint.

These are corrections applied after the image has been digitized and stored, and therefore are unable to deliver the highest quality result that could be achieved by optimizing or correcting the acquisition process in the first place.

However, acquiring the optimum quality image can be impractical – reasons

- low dose imaging – noisy
- intensity variations – irregularities of surfaces
- outliers – x-rays, dead pixels

Non-uniform illumination correction

Figure 4.58 Macro image (a) with nonuniform illumination. The background image (b) was recorded under the same conditions with the specimen removed from the copy stand. Subtracting the background image pixel-by-pixel from the original and expanding the contrast of the result produces a leveled image (c).

- Take an image from the background, and post-process the images - leveling

Fitting a background function

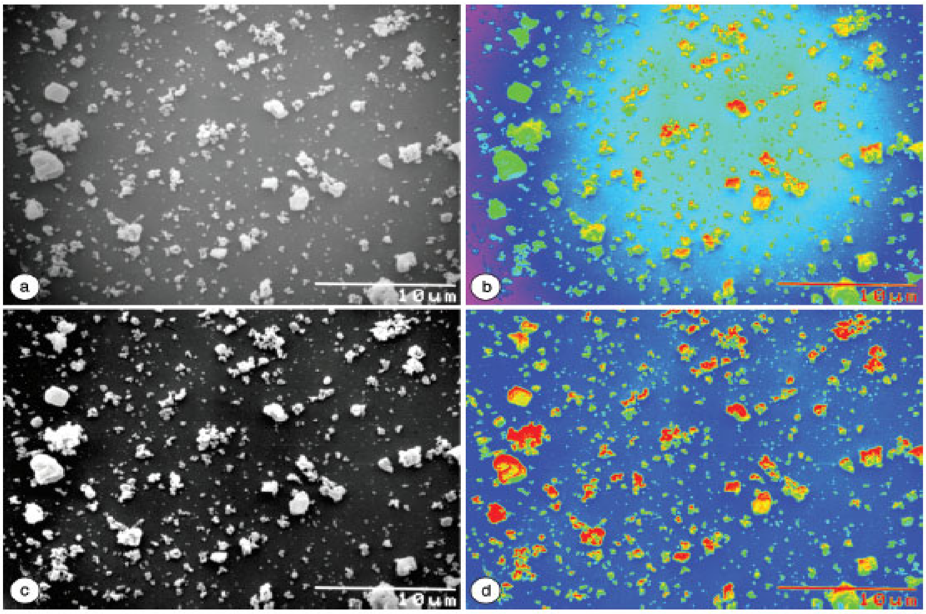


Figure 4.63 Leveling image contrast: (a) SEM image of particulates on a substrate; (b) false color applied to (a) to make the shading more visually evident; (c) image leveled by automatic polynomial fitting; (d) false color applied to (c).

- Background points are selected automatically after enhancement using color
- The variation of the background is approximated by a polynomial
- Image is divided in many regions, and darkest pixels are found in region

Image Enhancement

Reasons to enhance image

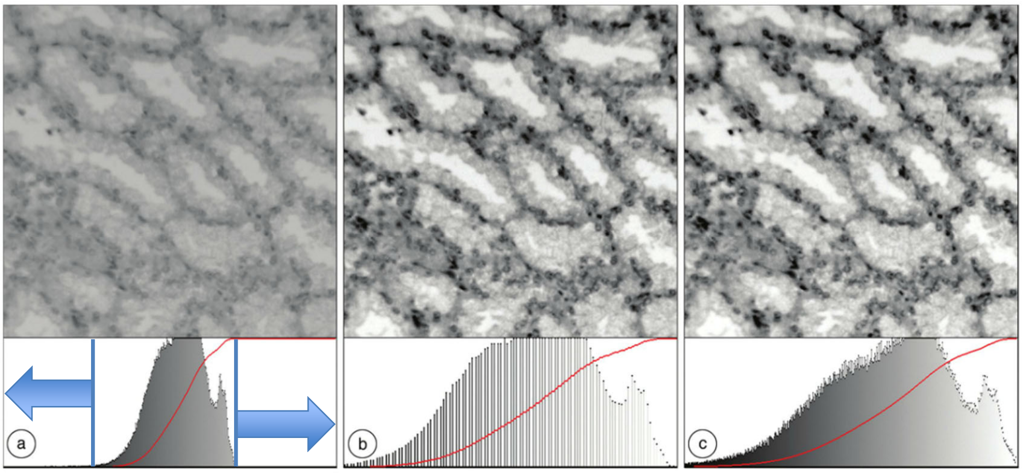
- Make the image easier to visually examine and interpret
- Apply methods based on the physics of image interpretation
- Improve printing/documenting of images
- Allow automatic methods to perform measurements (segmentation, binarization)

Contrast Expansion – Linear

Visibility of the structures present can be improved by stretching the contrast so that the values of the pixels are re-assigned to cover the entire available range

$$P' = 255 \cdot \frac{P - \text{Darkest}}{\text{Brightest} - \text{Darkest}}$$

raw image | contrast enhanced by *linear expansion* | contrast enhanced during acquisition



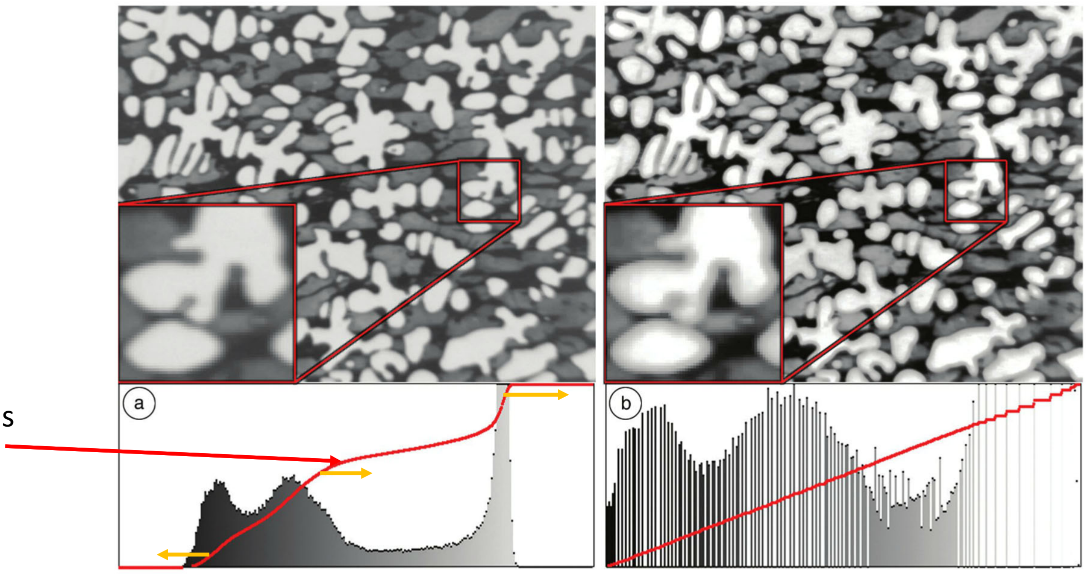
Linear contrast enhancement -> brightness and contrast, window levels

Histogram Equalization

The data are plotted as a cumulative curve which is the integral or summation of the pixel values. Then this curve is used as the transfer function

$$f(j) = \frac{255}{T} \sum_{i=0}^{j} N_i$$

ummed pixels



The rationale is to spread out the displayed brightness levels in the peak areas while compressing them in the valleys so that the same number of pixels in the display show each of the possible brightness levels

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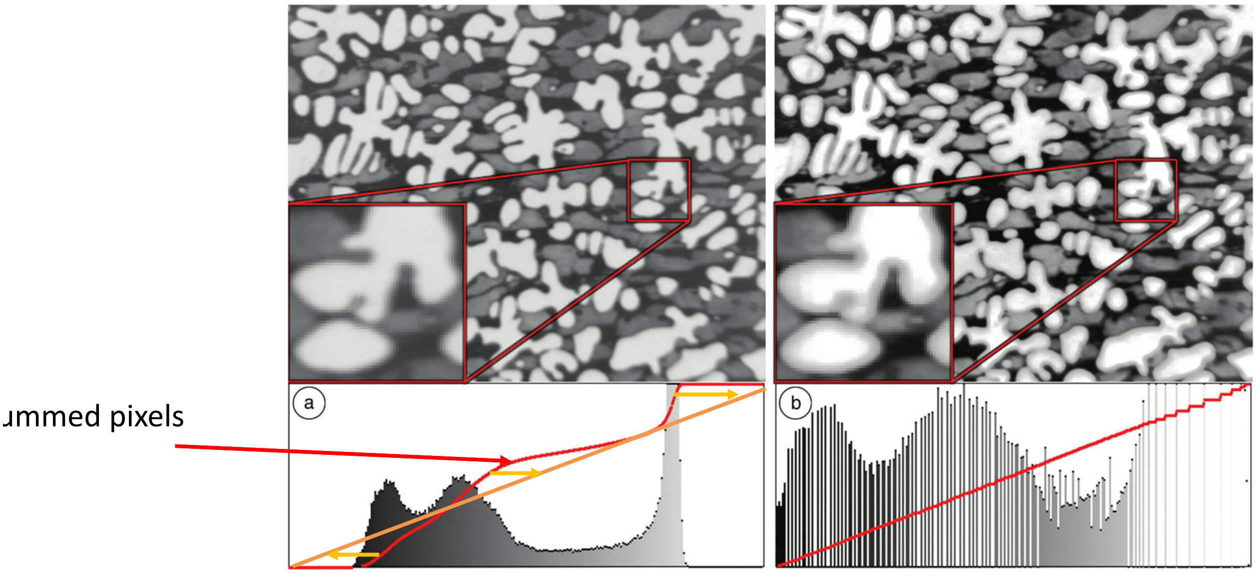
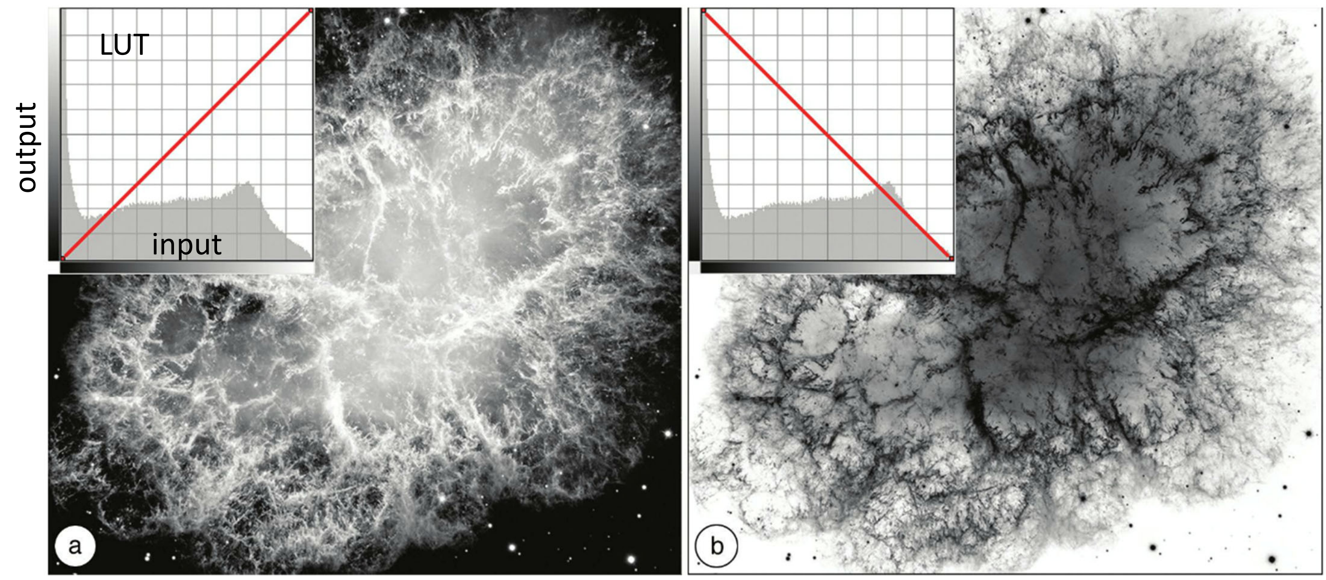


The rationale is to spread out the displayed brightness levels in the peak areas while compressing them in the valleys so that the same number of pixels in the display show each of the possible brightness levels

Examples – Linear Manipulation

raw image

contrast inverted image



- The transfer function is a straight line
- Reversing the plot makes the human vision to discern details better

LUT =Look up table

mapping original grey levels to new grey levels

Contrast Manipulation – *Non-Linear –Gamma Value*

The manipulation of pixel brightness can be described in terms *of a transfer function* relating the stored brightness value for each pixel to a displayed value

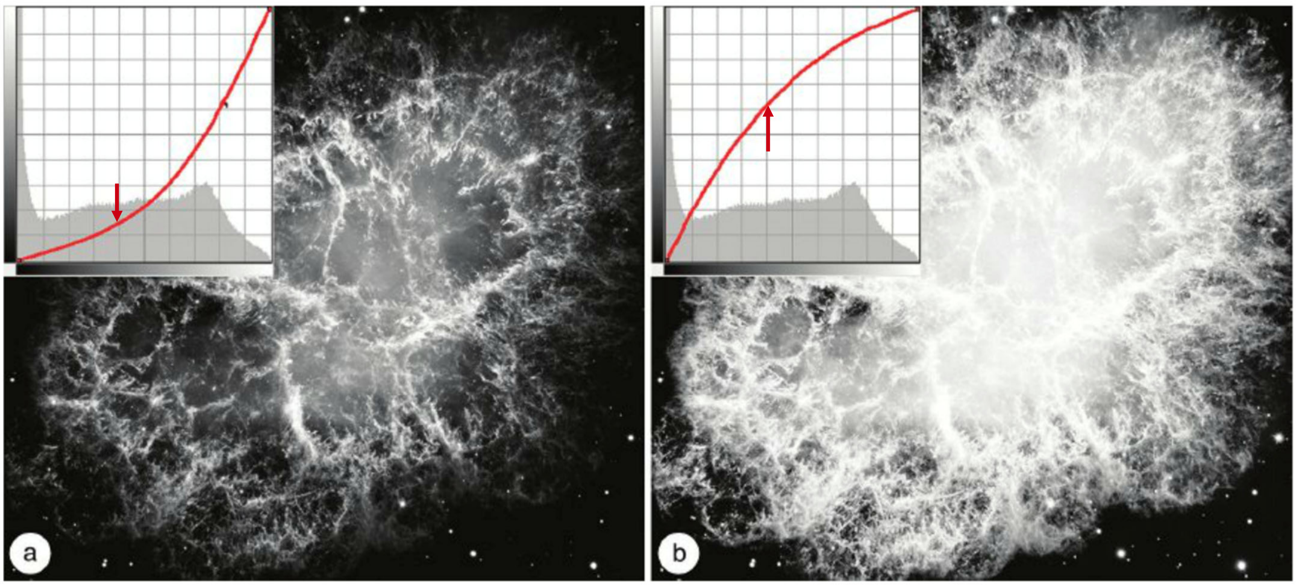
By altering the transfer function, the contrast can be expanded so that some of the image details become more visible at the expense of compressing the contrast for others

It is all about manipulating the *Gamma* value

$$\left(\frac{\text{Display}}{255}\right) = \left(\frac{\text{Original}}{255}\right)^{\text{Gamma}}$$

Examples – Non-Linear Manipulation

gamma > 1 gamma <1

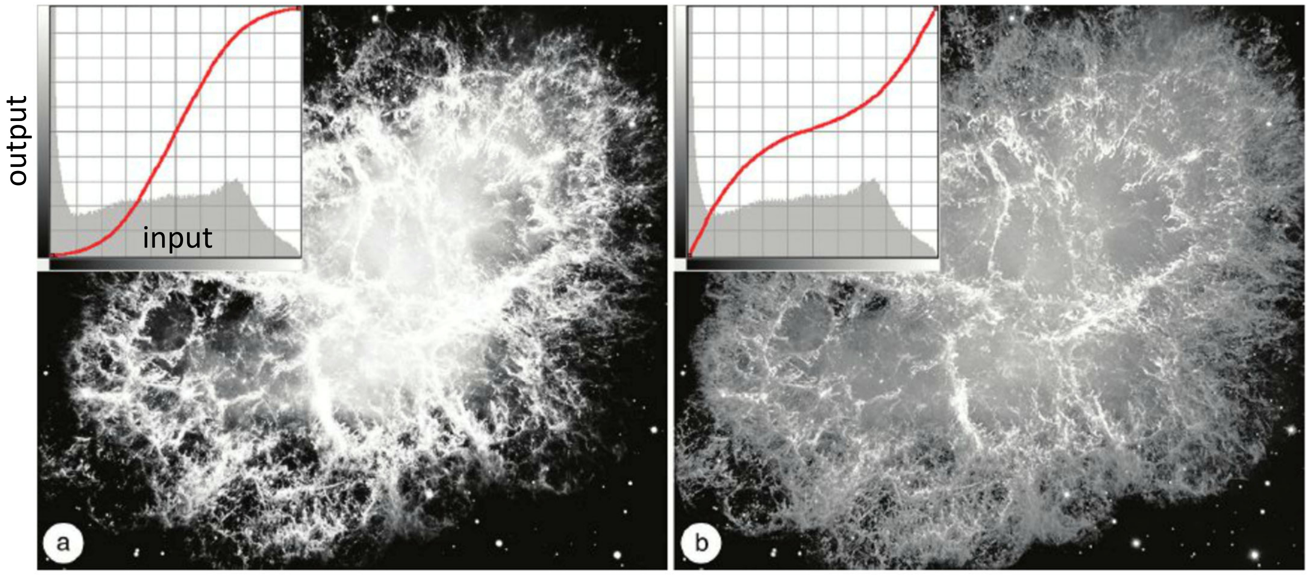


- (a) expansion for bright values – more details in the central area
- (b) expansion for the dark values – compresses the values in the bright areas

Examples – Non-Linear Manipulation

expansion for middle grey values

expansion for extreme grey values

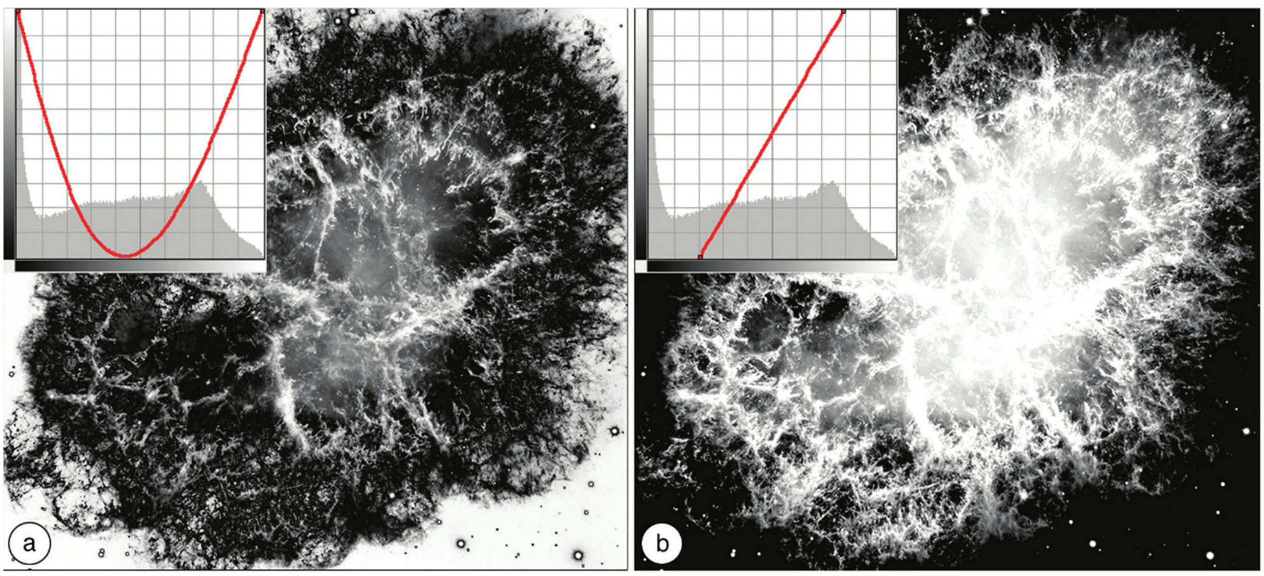


- Arbitrarily created transfer function can be more important or interesting
- (a) increased contrast in positive and negative regions
- (b) clipping extreme values to white and black expands the contrast in the middle

Examples – Contrast Manipulation

partial reversal

clipping of histogram



Ultimately this is a flexible tool – the problem is that it is manual and depends on your judgment to determine which details are important – be aware of conscious or unconscious misconceptions on how the image should look like!

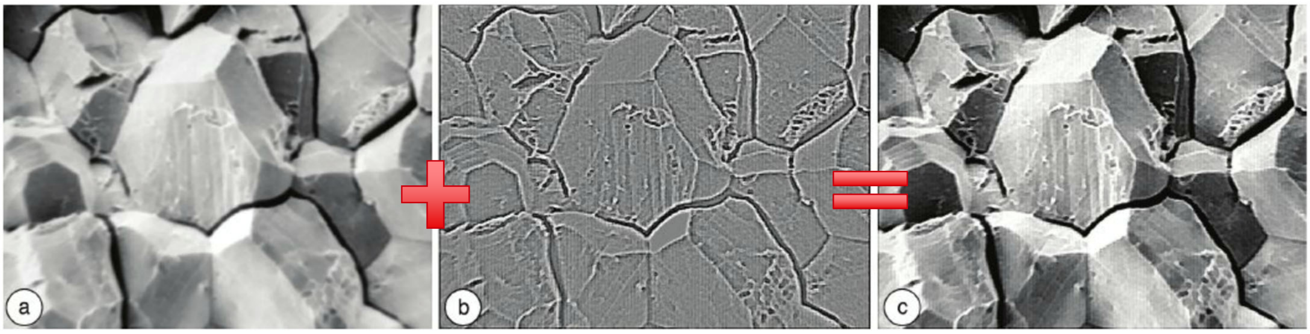
Filters: Laplacian Sharpening

Local equalization of image contrast produces an increase in local contrast at boundaries, which has the effect of making easier for the viewer to see

a 3x3 Laplacian convolution operator would be:

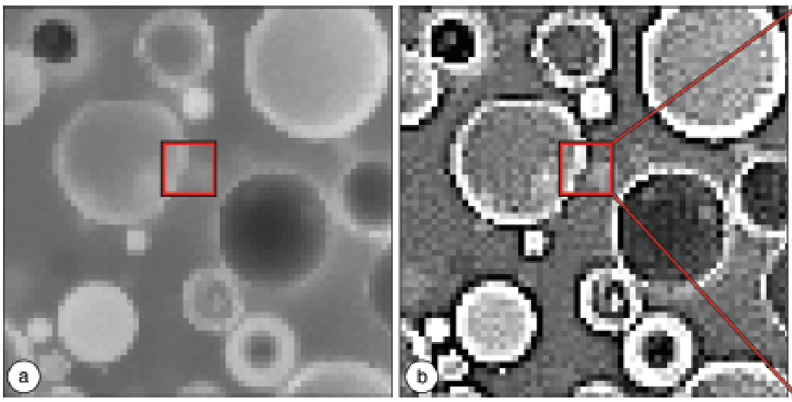
$$\begin{bmatrix} -1 & -1 & -1 \\ -1 & +8 & -1 \\ -1 & -1 & -1 \end{bmatrix} + \begin{bmatrix} 0 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 0 \end{bmatrix} = \begin{bmatrix} -1 & -1 & -1 \\ -1 & +9 & -1 \\ -1 & -1 & -1 \end{bmatrix}$$

SEM raw image | Laplacian operator | sharpening (a+b)



Unlike smoothing kernels, this operator represents the second derivative and it is not a weighted kernel, it highlights DIFFERENCES

Sharpening kernel example



Original Values							
119	124	145	131	91	86	84	79
118	126	145	134	92	89	86	86
124	132	145	134	100	96	93	87
126	134	139	133	107	106	106	106
134	139	134	113	106	113	114	108
139	139	115	102	109	110	112	109
142	145	113	101	100	104	109	106
141	145	102	95	92	92	102	101

After Sharpening							
125	84	240	226	6	75	83	56
106	85	239	228	2	80	73	96
125	127	232	214	23	83	74	41
96	129	190	231	60	123	136	165
136	194	200	82	66	145	154	122
139	192	55	36	126	129	142	111
152	255	77	74	95	112	140	111
192	255	49	70	71	61	122	101

Figure 5.23 *Operation of the 3 × 3 sharpening filter: (a) fragment of an original image (an epoxy containing bubbles); (b) after application of the filter the edges are delineated by bright and dark borders. The pixel values are shown for the outlined region. On the dark side of the edge, pixel values are reduced (blue), and on the bright side, they are increased (red).*

- The effect of the filter is to make the pixels on the dark side of an edge darker and those on the bright side brighter, thus increasing the edge contrast

Non-symmetric Filters: Edge Detection

The process of locating boundaries with arbitrary orientation in two dimensional images -> it refers to taking first derivatives of brightness in different orientations

(Robinson)

$$\begin{bmatrix} +1 & 0 & -1 \\ +1 & 0 & -1 \\ +1 & 0 & -1 \end{bmatrix} \quad \begin{bmatrix} +1 & +1 & 0 \\ +1 & 0 & -1 \\ 0 & -1 & -1 \end{bmatrix}$$

(Sobel)

$$\begin{bmatrix} +1 & 0 & -1 \\ +2 & 0 & -2 \\ +1 & 0 & -1 \end{bmatrix} \quad \begin{bmatrix} +2 & +1 & 0 \\ +1 & 0 & -1 \\ 0 & -1 & -2 \end{bmatrix}$$

(Prewitt)

$$\begin{bmatrix} +1 & -1 & -1 \\ +1 & +2 & -1 \\ +1 & -1 & -1 \end{bmatrix} \quad \begin{bmatrix} +1 & +1 & -1 \\ +1 & +2 & -1 \\ -1 & -1 & -1 \end{bmatrix}$$

(Kirsch)

$$\begin{bmatrix} +5 & -3 & -3 \\ +5 & 0 & -3 \\ +5 & -3 & -3 \end{bmatrix} \quad \begin{bmatrix} +5 & +5 & -3 \\ +5 & 0 & -3 \\ -3 & -3 & -3 \end{bmatrix}$$

and so forth, to respond to edges in various orientations.

Filters: Edge Detection

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+ -

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and so forth, to respond to edges in various orientations.

Filters: Sobel Edge Detection

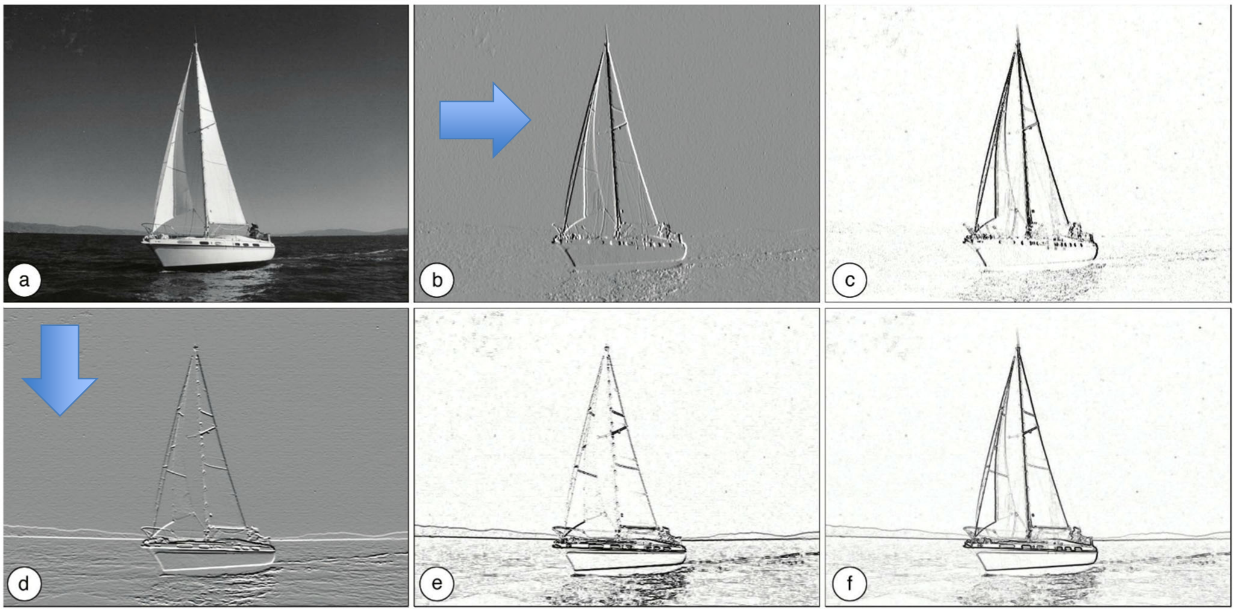


Figure 5.38 *Directional derivatives and the Sobel edge operator: (a) original image; (b) horizontal derivative ("embossed" image); (c) absolute value of the horizontal derivative; (d) vertical derivative ("embossed" image); (e) absolute value of the vertical derivative; (f) combining the derivative magnitudes by squaring, adding, and taking the square root (Sobel operator).*

Filters: Sobel Edge Detection – How it works

The operator consists of a pair of 3 × 3 convolution kernels; One kernel is simply the other rotated by 90° . Two 3 × 3 convolution kernels (shown below) are used to generate vertical and horizontal derivatives. The final image is produced by combining the two derivatives using the square root of the sum of the squares.

-1	0	+1
-2	0	+2
-1	0	+1

Gx

+1	+2	+1
0	0	0
-1	-2	-1

Gy

P_1	P_2	P_3
P_4	P_5	P_6
P_7	P_8	P_9

$$\mathbf{G} = \sqrt{\mathbf{G}_x^{\,2} + \mathbf{G}_y^{\,2}}$$

$$|G| = |Gx| + |Gy|$$

Using this information, we can also calculate the gradient's direction:

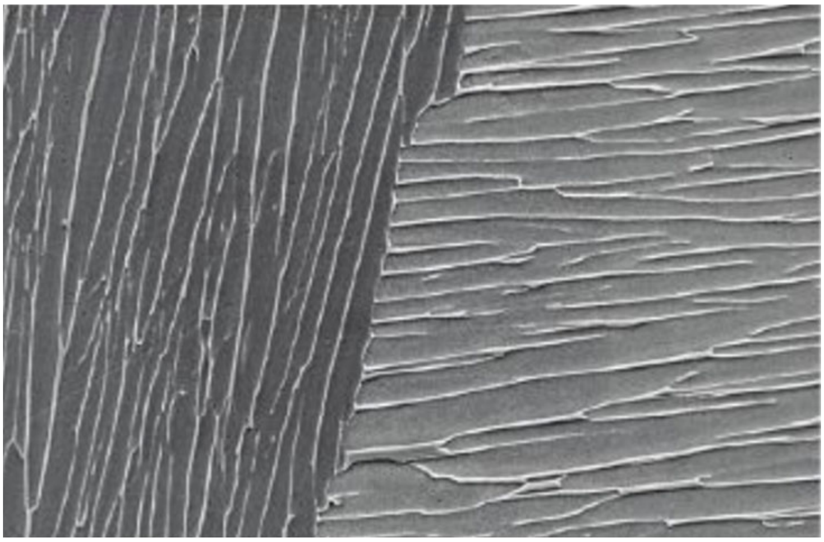
$$\mathbf{\Theta} = \operatorname{atan2}(\mathbf{G}_y, \mathbf{G}_x)$$

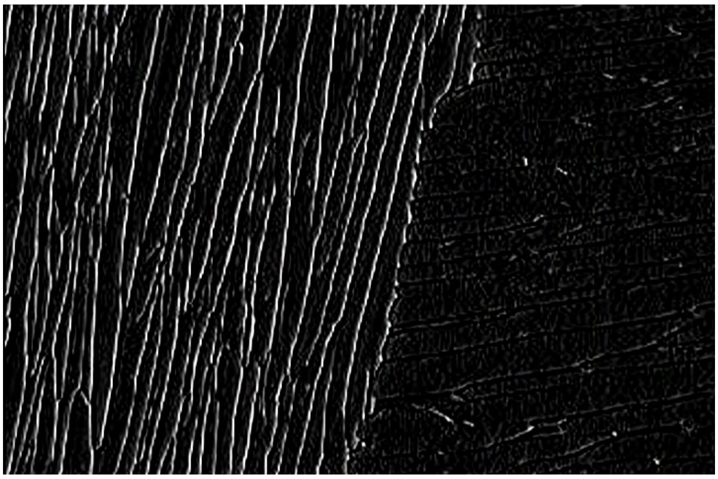
Gradient: Amplitude **G** and direction θ

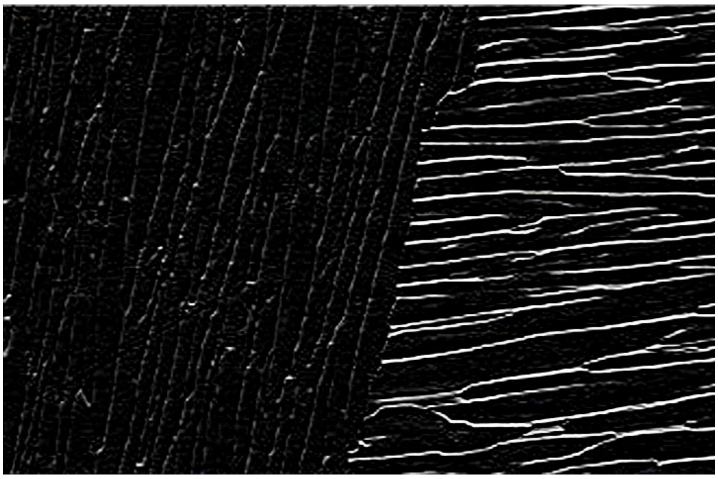
$$|G| = |(P_1 + 2 \times P_2 + P_3) - (P_7 + 2 \times P_8 + P_9)| + |(P_3 + 2 \times P_6 + P_9) - (P_1 + 2 \times P_4 + P_7)|$$

In ImageJ this function is under "Find Edges"

$$Gx = \begin{matrix} -1 & 0 & 1 \\ -2 & 0 & 2 \\ -1 & 0 & 1 \end{matrix}$$

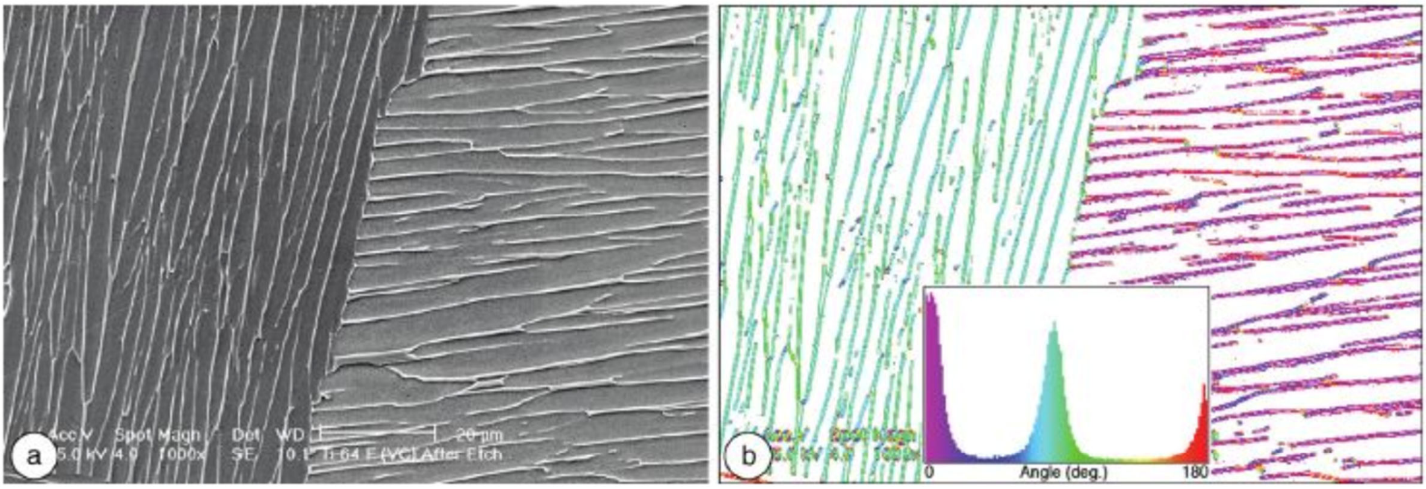






$$Gy = \begin{matrix} -1 & -2 & -1 \\ 0 & 0 & 0 \\ 1 & 2 & 1 \end{matrix}$$

Sobel Edge Detection – Example 1



gure 5.42 Lamellar structure in a titanium alloy: (a) SEM image; (b) edges selected by magnitud the Sobel gradient and color-coded with orientation, with the histogram of edge orientations f 0 to 180 degrees. (SEM image courtesy of Hamish Fraser, Ohio State University.)

- Setting a threshold value at the magnitude of the vector |G| > G_0 and keeping the angle information defined by (Gx, Gy) for those pixels above the threshold

Sobel Edge Detection – Example 1

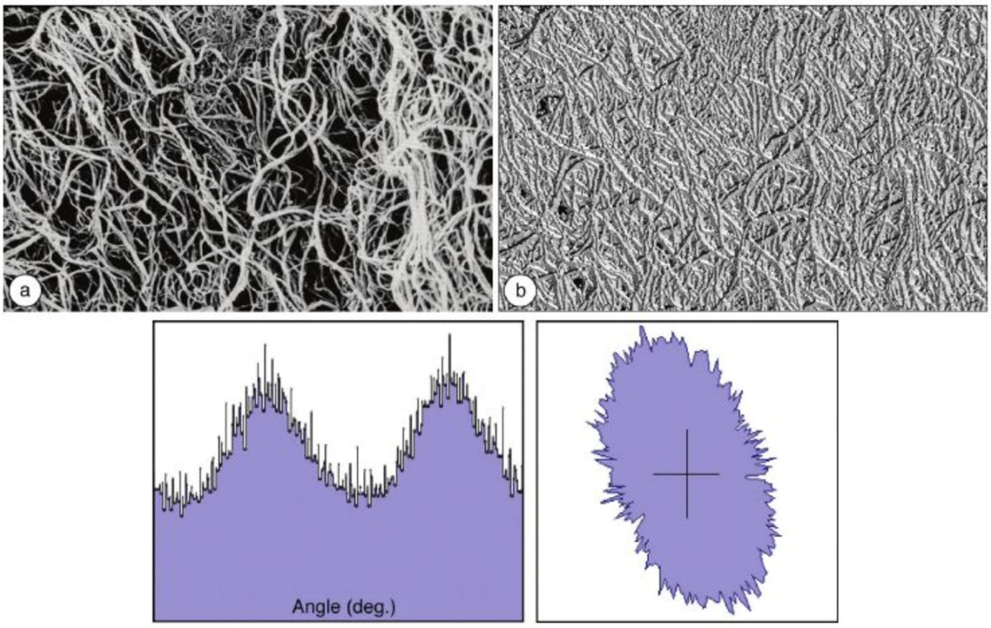


Figure 5.43 Measurement of fiber orientation: (a) image of collagen fibers; (b) the Sobel orientation values assigned to pixel brightness. The histograms show the values in (b) from 0 to 360 degrees and as a rose plot.

- The efficiency with which the Sobel operator can be applied make it a very useful technique for characterizing the anisotropy of lines and edges

Processing Images in Frequency Space

Fourier Transform Basics

ually, the mathematical background of a Fourier Transform begins with a one-dimensional waveform and then ɔands to two dimensions (image).

urier's theorem (1822) states that it is possible to construct any well-behaved one-dimensional function *f(x)* as a nmation of a series of sine and cosine terms of increasing frequency, *F(u)*

$$F(u) = \int_{-\infty}^{+\infty} f(x) \cdot e^{-2\pi iux} dx$$

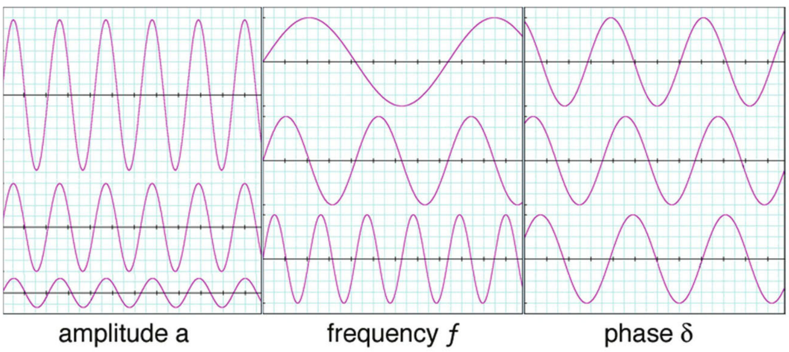


Figure 6.1 Varying the parameters for the sine function $y = a \cdot \sin(f \cdot 2\pi x + \delta)$, where *a* is the amplitude, *f* is the frequency, and δ is the phase.

Origin: application in signal processing

Fourier Transform Basics

In the Fourier domain image, each point represents a particular frequency contained in the spatial domain image

The image in the spatial and Fourier domain are of the same size

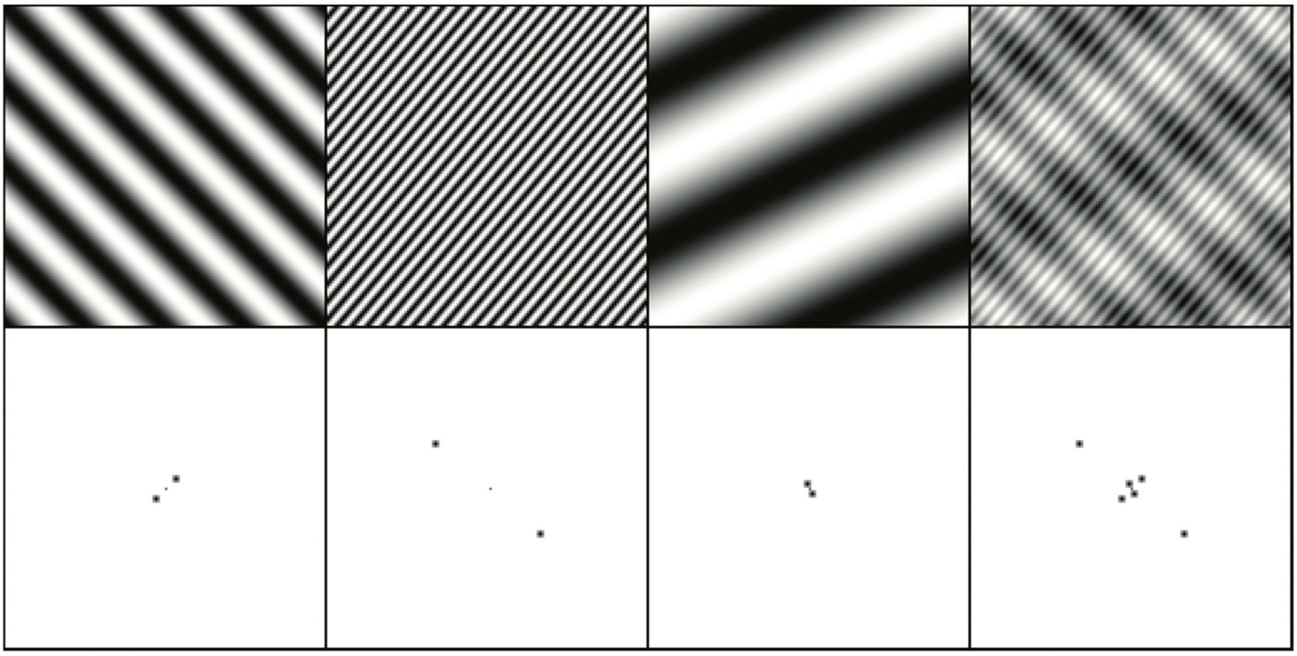
For a square image of size N x N, the two dimensional FFT is:

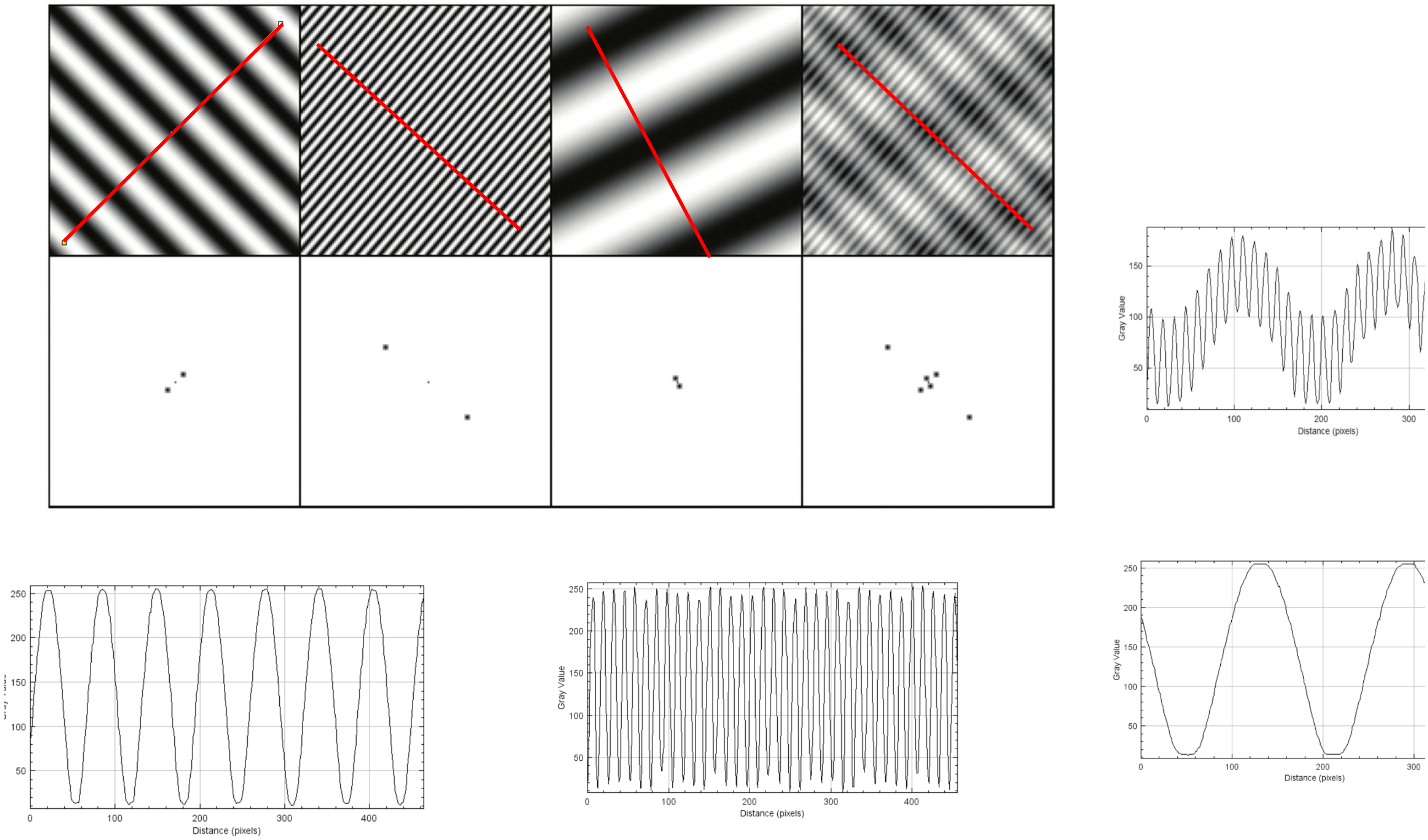
$$F(k,l) = \sum_{i=0}^{N-1} \sum_{j=0}^{N-1} f(i,j)\, e^{-\iota 2\pi \left(\frac{ki}{N} + \frac{lj}{N}\right)}$$

the basis functions are sine and cosine waves with increasing frequencies

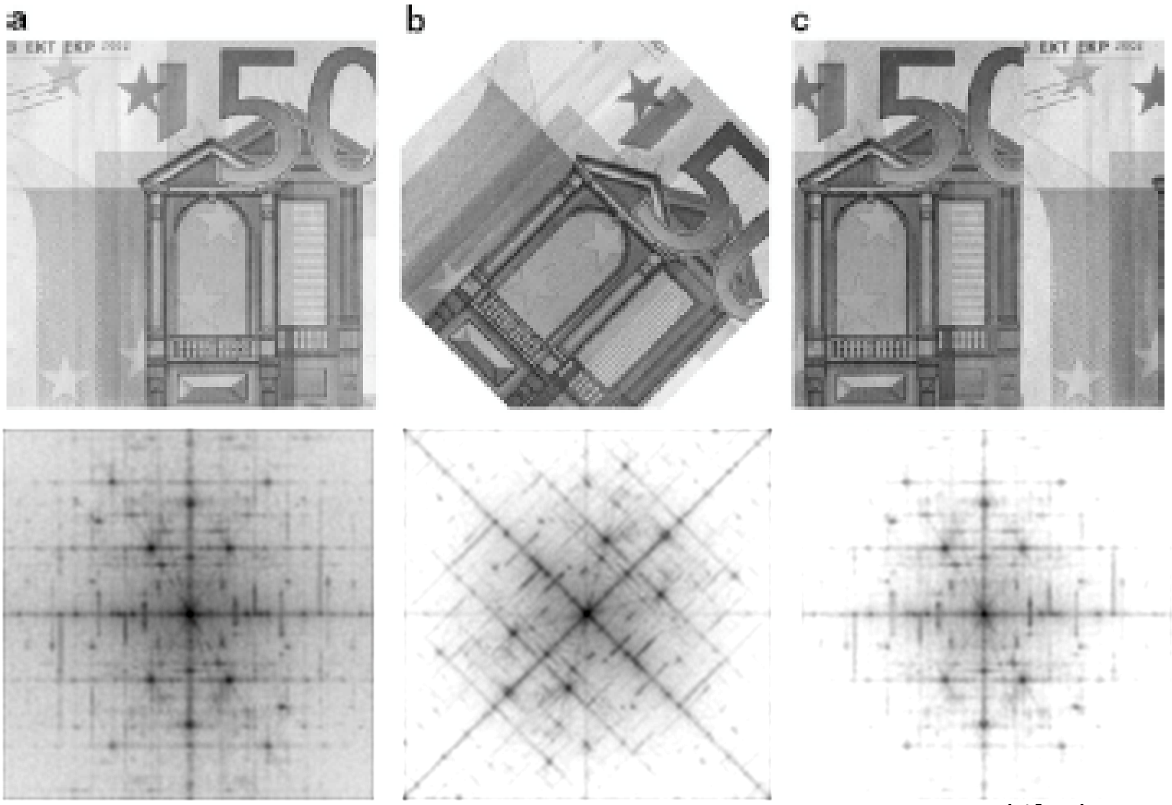
Two dimensional Fourier Transform

The two dimensional power spectra employ polar coordinates – the frequencies are plotted radially from the center





Two dimensional Fourier Transform - Properties



- Image rotated
- Image shifted

Inverse Fourier Transform, Fourier Filtering

The information content of a real image and its Fourier transform is the same – the one defines the other

As you can calculate the Fourier transform of an image, you can also calculate an image from an FFT

$$f(a,b) = \frac{1}{N^2} \sum_{k=0}^{N-1} \sum_{l=0}^{N-1} F(k,l)\, e^{\iota 2\pi\left(\frac{ka}{N}+\frac{lb}{N}\right)}$$

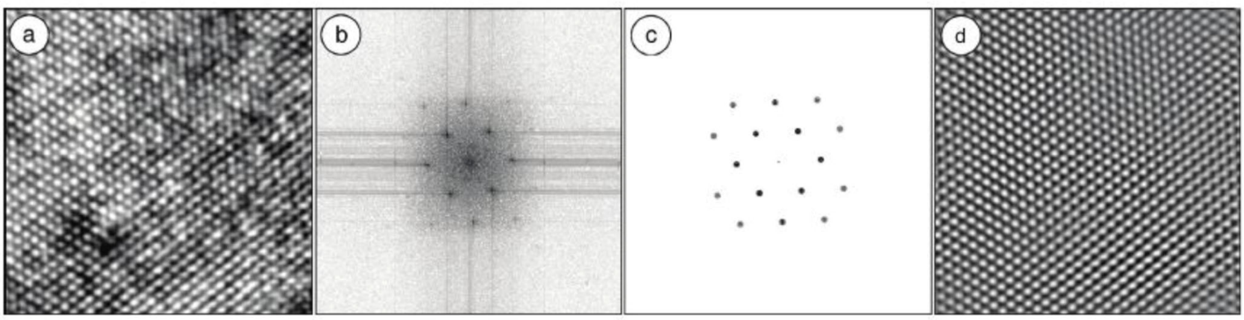
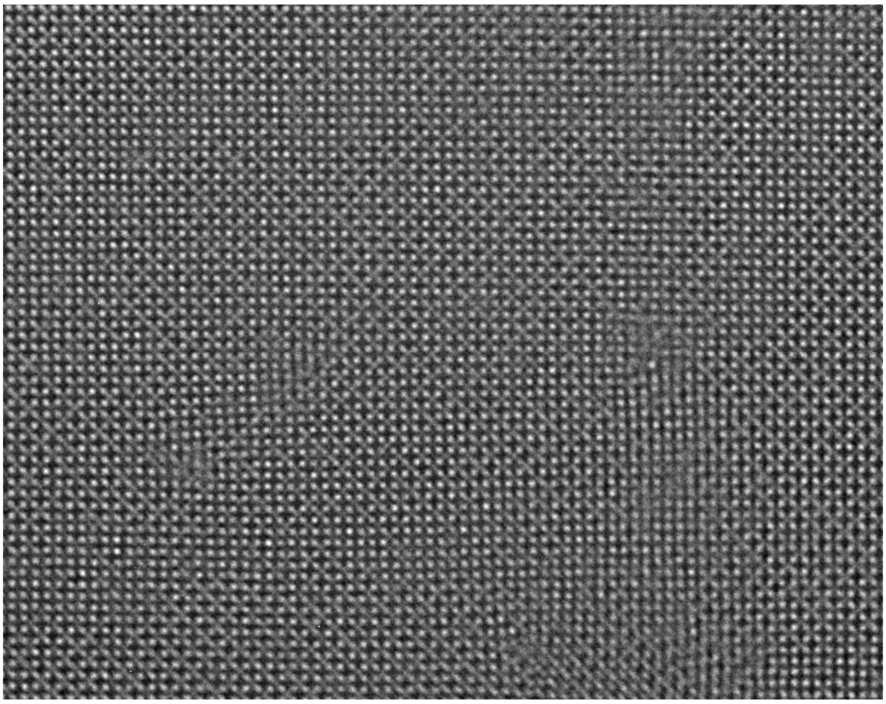


Image | FFT | "cleaned FFT" | inverse FFT

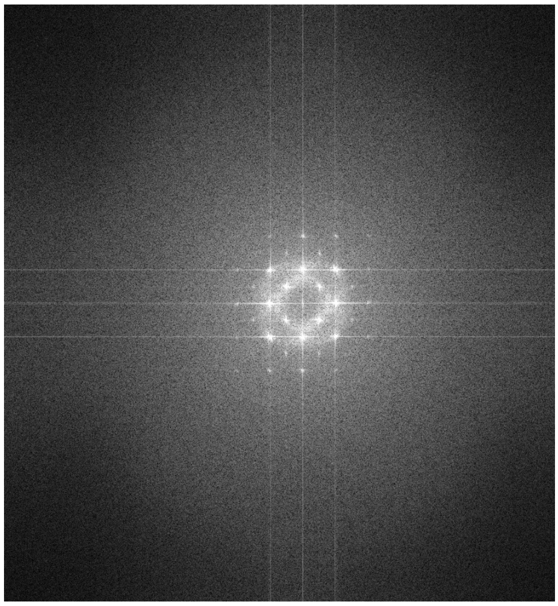
Figure 6.12 *(a) High-resolution TEM image of atomic lattice in silicon (a), with (b) the frequency transform. (c) Isolating just the periodic frequencies and (d) retransforming shows just the atom positions.*

The Fourier domain image has a much greater range (pixel values) than the image in the spatial domain. Hence, to sufficiently accurate, its values are usually calculated and stored in float values.

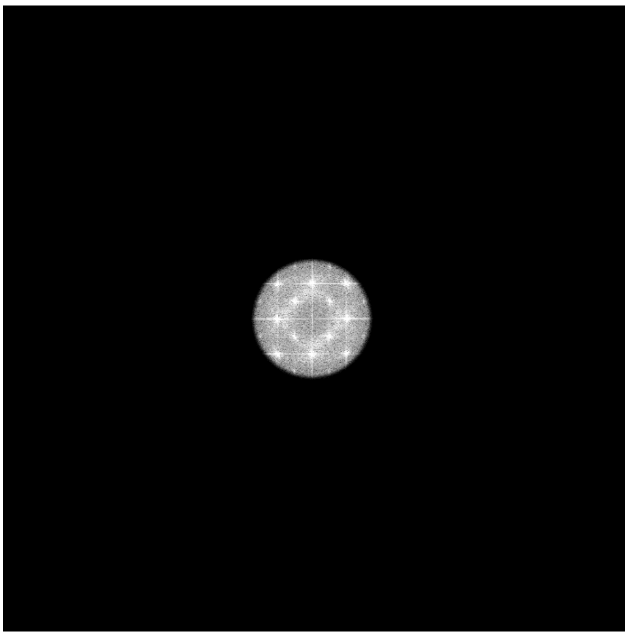
High-Resolution TEM image and its FFT



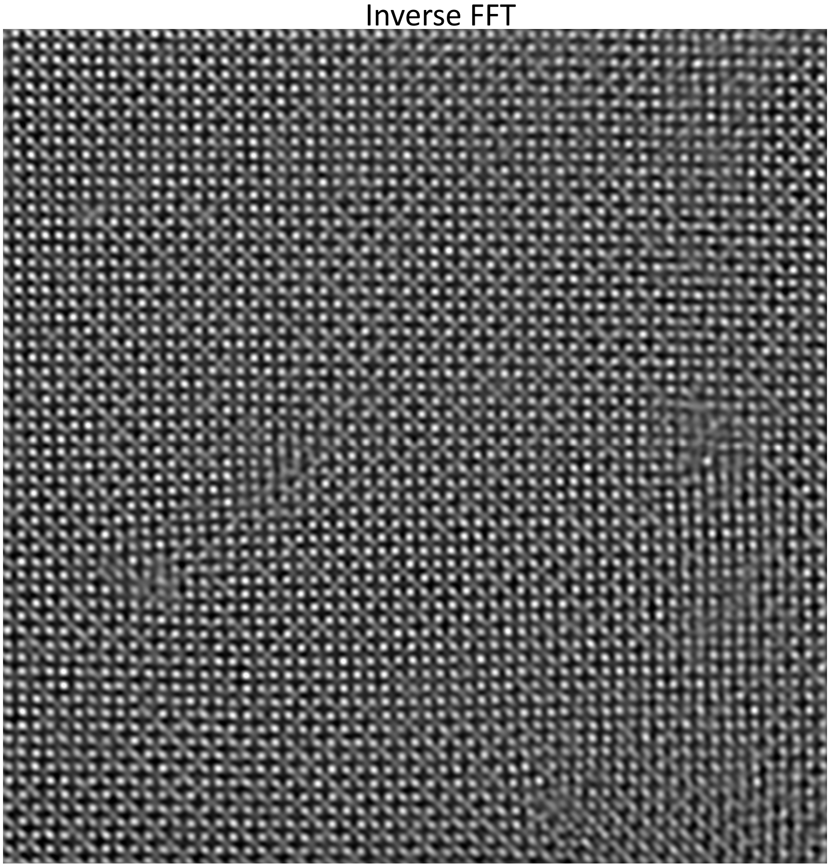
FFT



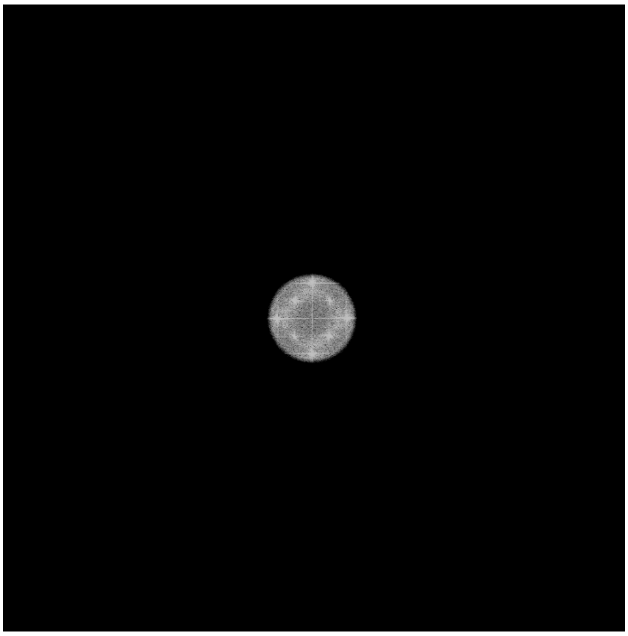
FFT “cleaned” up



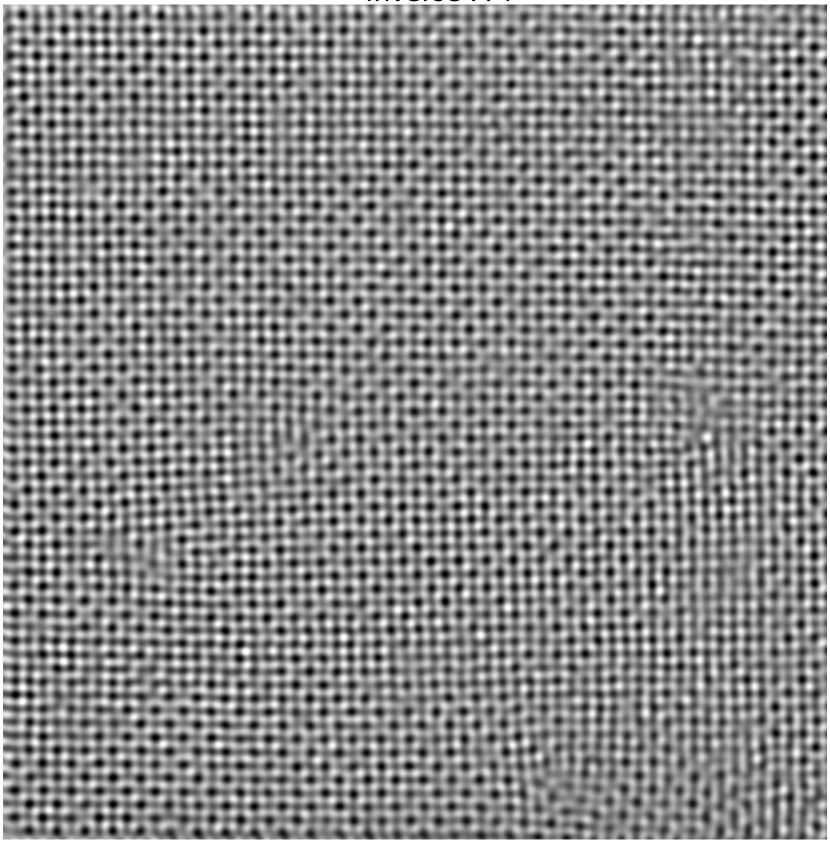
Inverse FFT

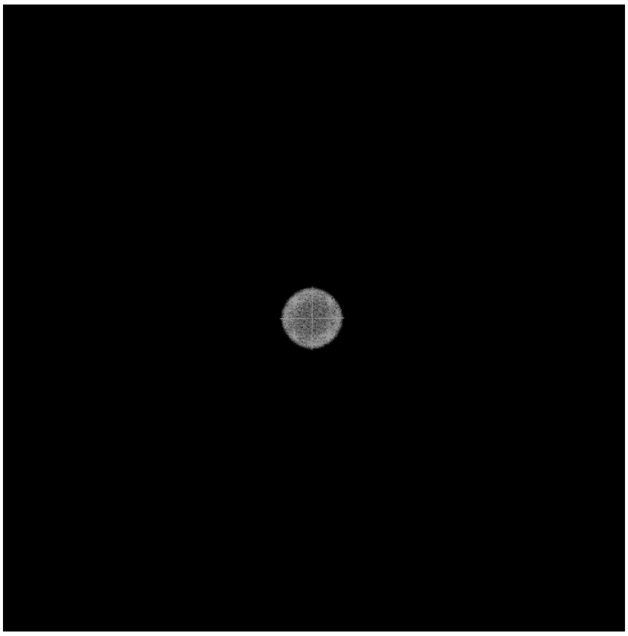


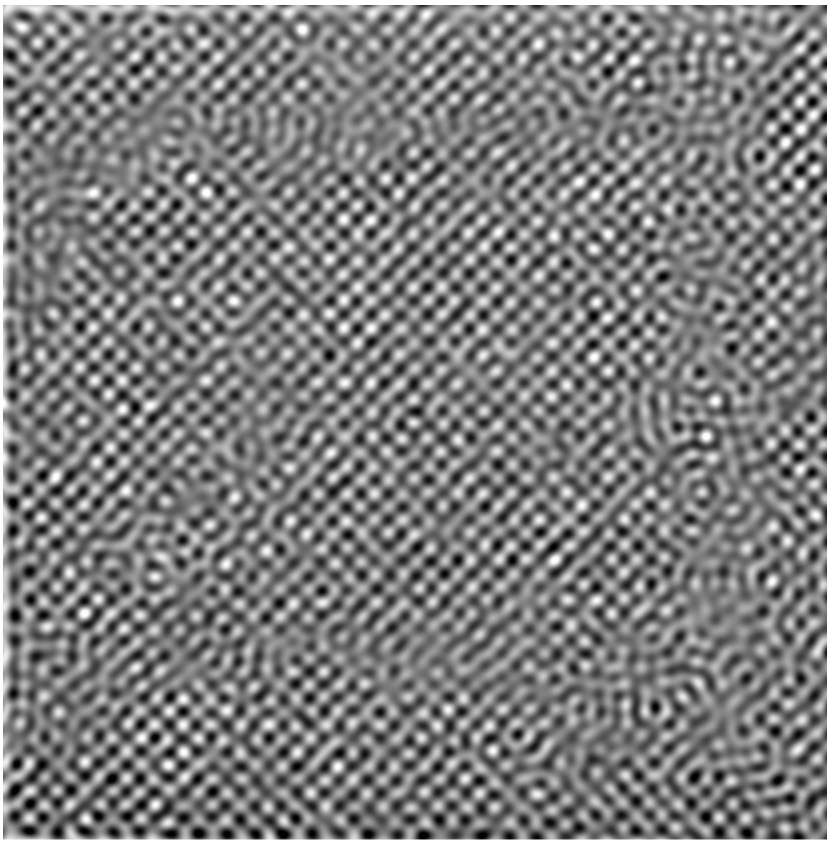
FFT "cleaned" up

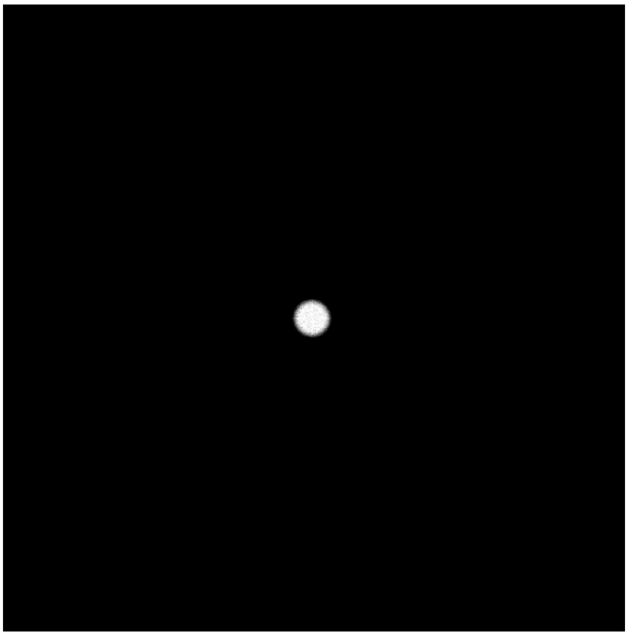
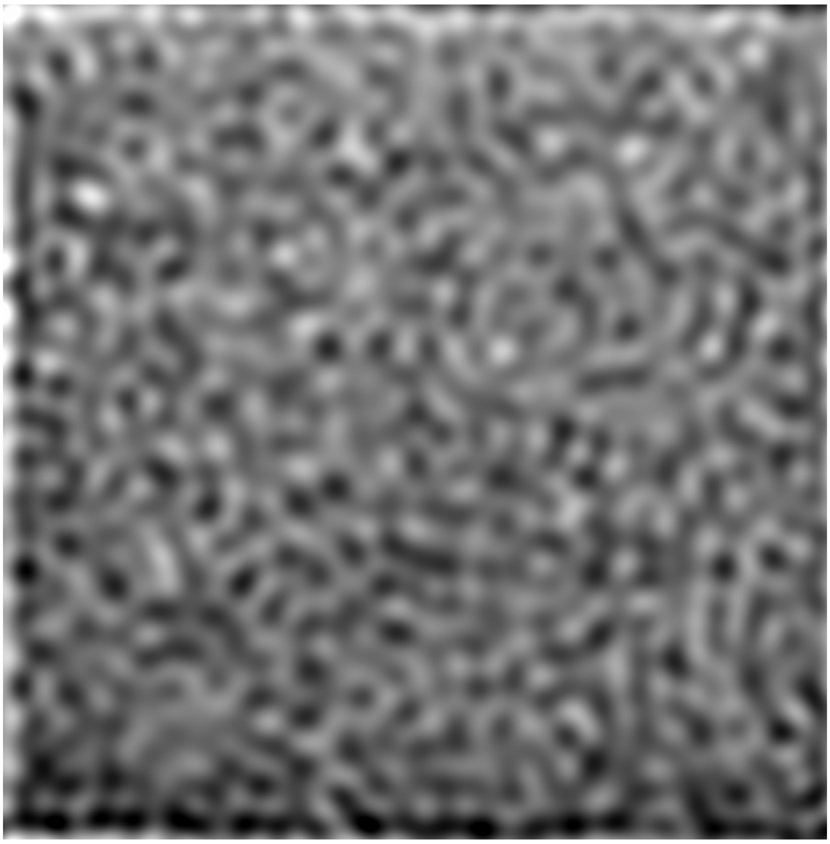
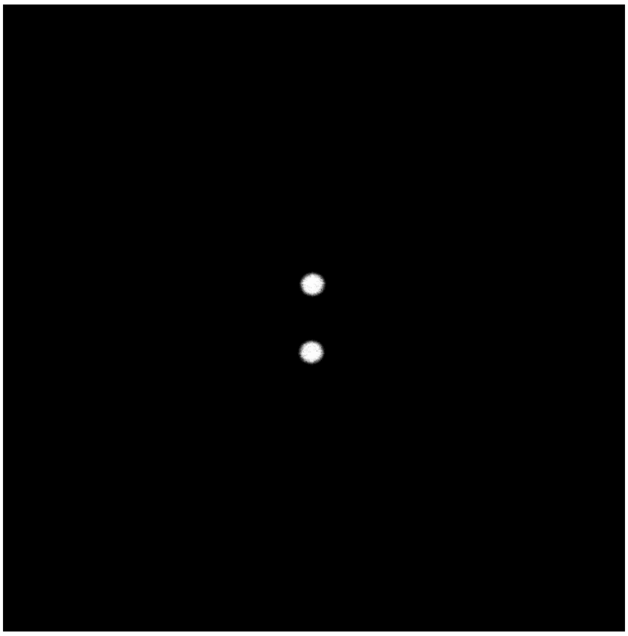
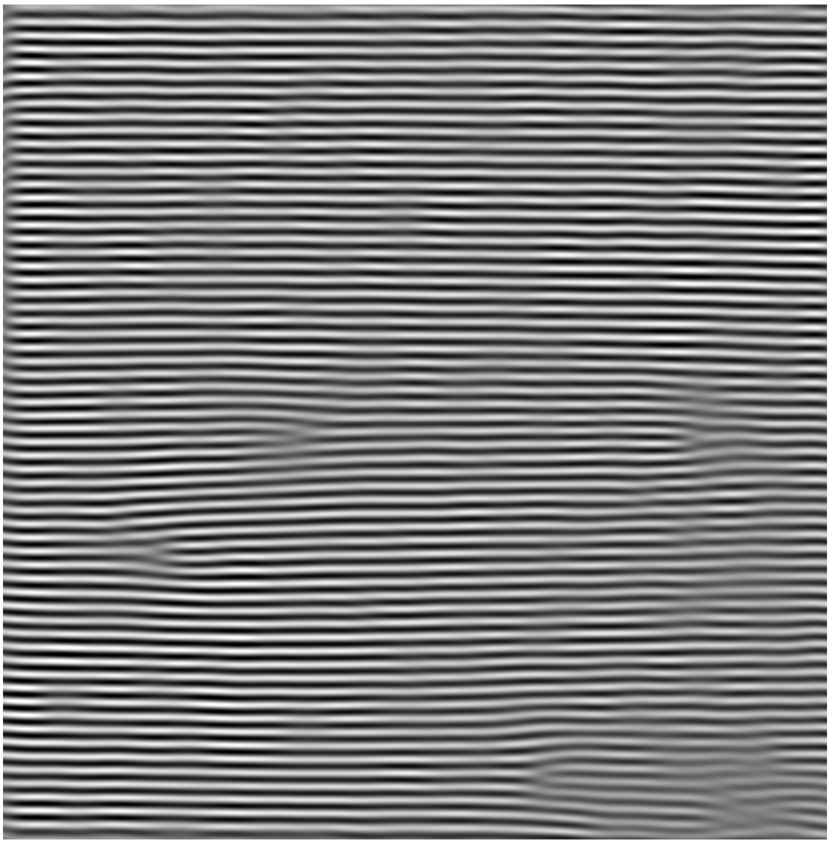


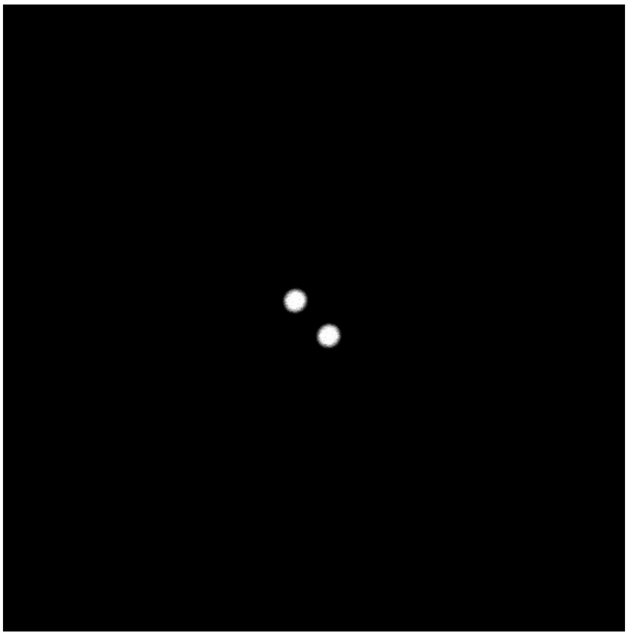
Inverse FFT

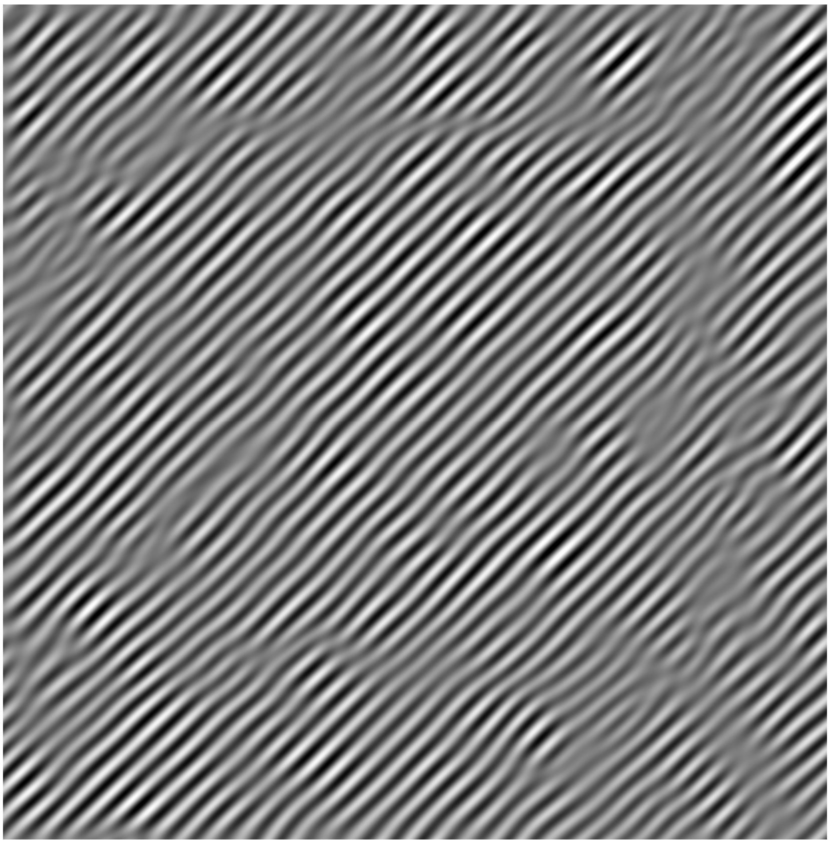








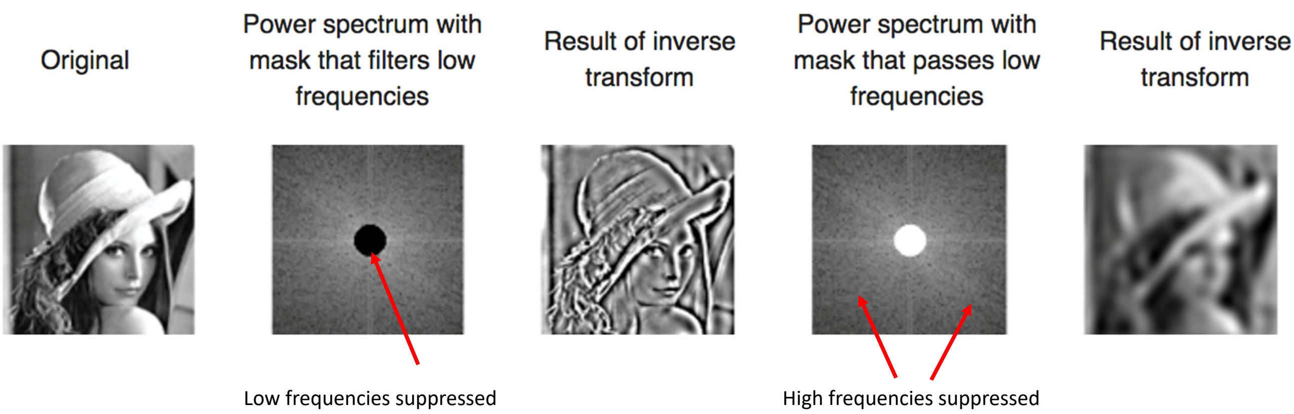


Frequency Filtering

The image is Fourier transformed, multiplied by the filter function and then re-transformed to the spatial domain. Attenuating higl frequencies results in a smoother image in the spatial domain, attenuating low frequencies enhances the edges

There are three kinds of filters: *lowpass*, *highpass*, *bandpass* filters

Lowpass: attenuates high frequencies and retains low frequencies -> [smoothing filter]
Highpass: yields edge enhancement of edge detections because edges contain mainly high frequencies
Bandpass: attenuates very low and very high frequencies but retains middle range



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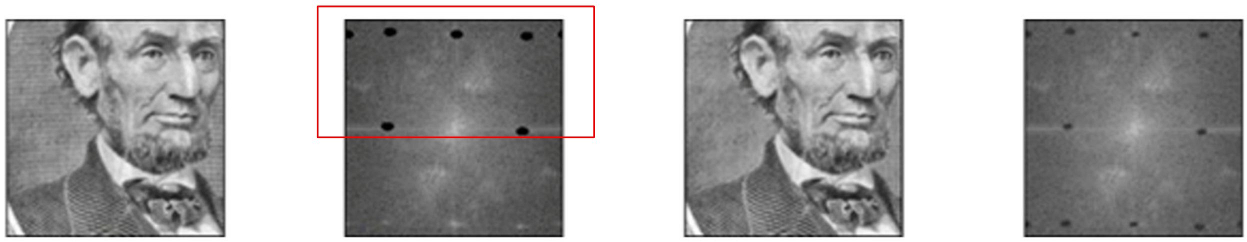
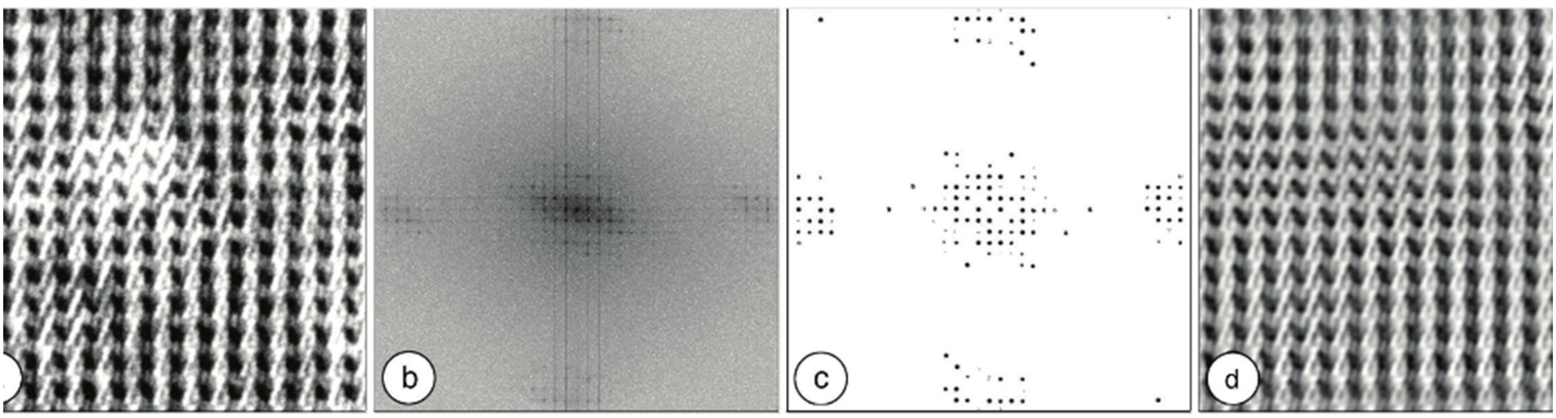
Original | Edited power spectrum | Inverse transform | Power spectrum after filtering



Selection of Periodic Information – Applying a Mask

original | FFT | filtered to construct a mask | inverse FFT with mask

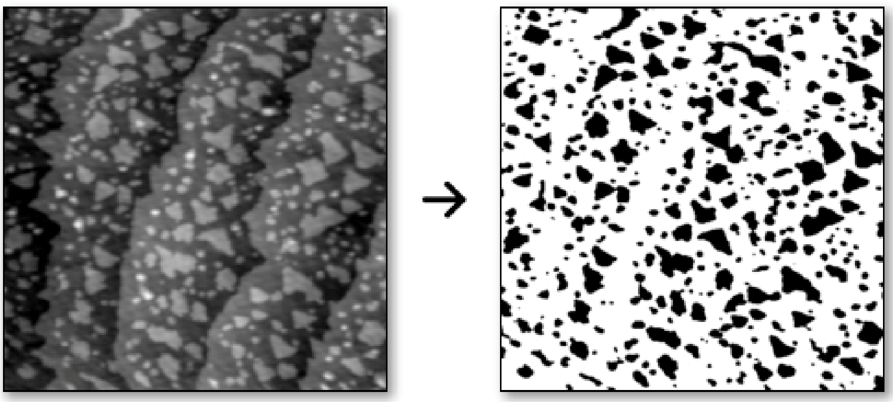


- The Fourier transform has peaks or spikes that represent the periodic structure
- Using a top hat filter with size just large enough to cover the spikes, a mask is constructed to to select a small region around each of the periodic spots

Segmentation & Thresholding

Thresholding concept

Selecting objects or features within an image by defining a range of brightness values in the original image – this selection process is called thresholding

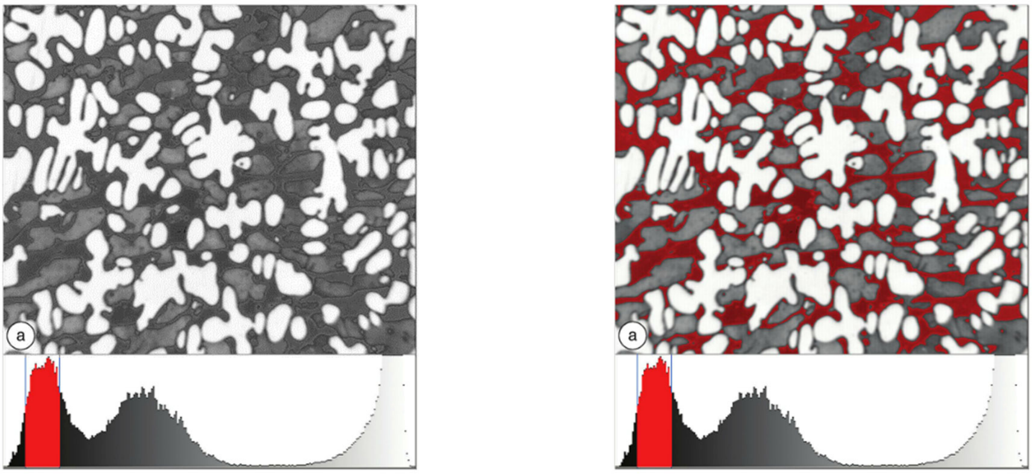


original image   thresholded

It may require *pre-processing* for optimal results
The output is a binary image representing the segmentation

Thresholding basics

Thresholding with the histogram means that a peak indicates that many pixels have nearly the same brightness, and that consequently they may represent the same structure or type of object



It proceeds with the assumption that a peak in the histogram corresponds to a structure in the image

Thresholding basics

Most of the times, the objects of interest do not share a single brightness or color value, nor a common range of those values. But if the background around the features, or the surface on which the objects lie, is fairly uniform color or brightness, it may be practical to select that by thresholding.

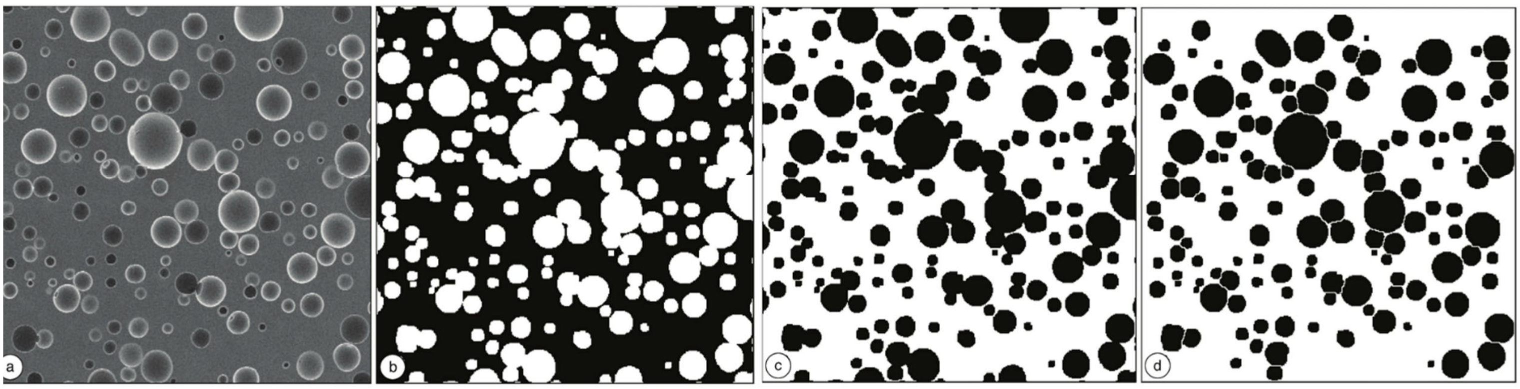
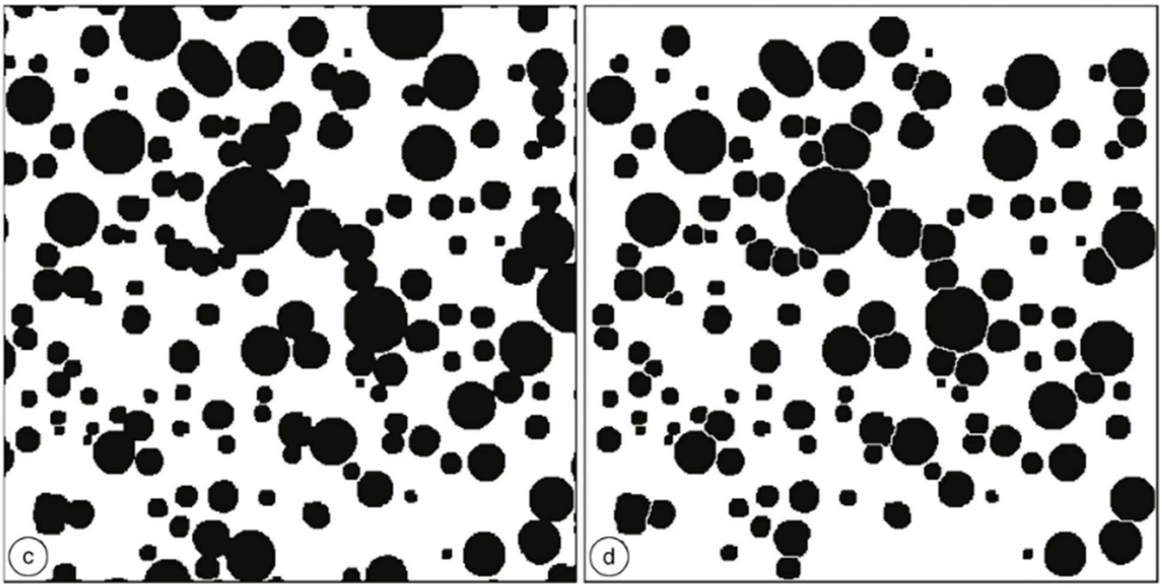


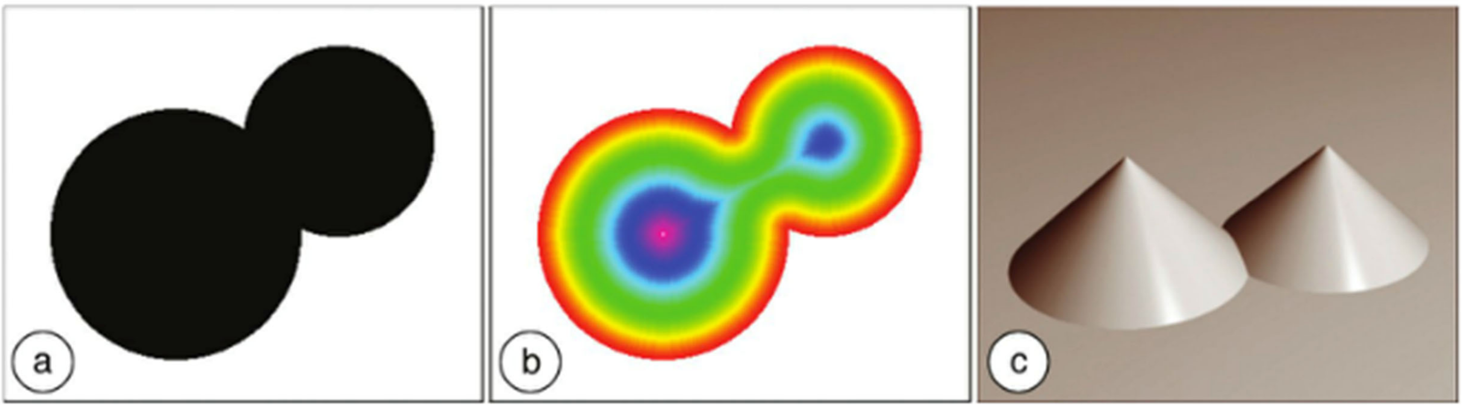
Figure 7.7 *Thresholding the background: (a) image of pores in epoxy resin; (b) thresholded intermediate gray background pixels and eliminating isolated regions that do not connect to the image boundaries; (c) inverted image delineating the pores; (d) a watershed applied to separate the measurable pores.*

Separation of touching particles by "watershedding"



Watersheding

Watershed segmentation is a way of automatically separating or cutting apart particles that touch. It works best f
smoothed objects that don't overlap too much and it applies to binary images only



It proceeds with a complicated algorithm that involves
erosion: removes pixels from features in an image by turning pixels OFF -> the ones that are touching
dilation: adds pixels -> can fill holes
euclidean distance map: every pixel is assigned a value that is its distance from the nearest background pixel

Watersheding

The assumption is that the features are sufficiently convex, i.e. the euclidean distance map has only one peak in each feature

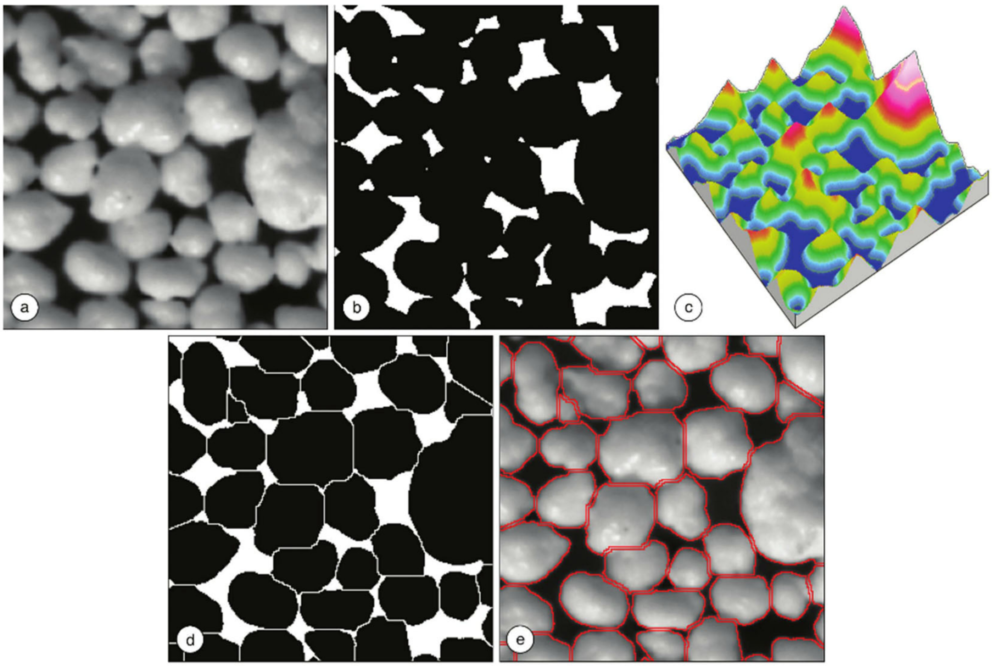
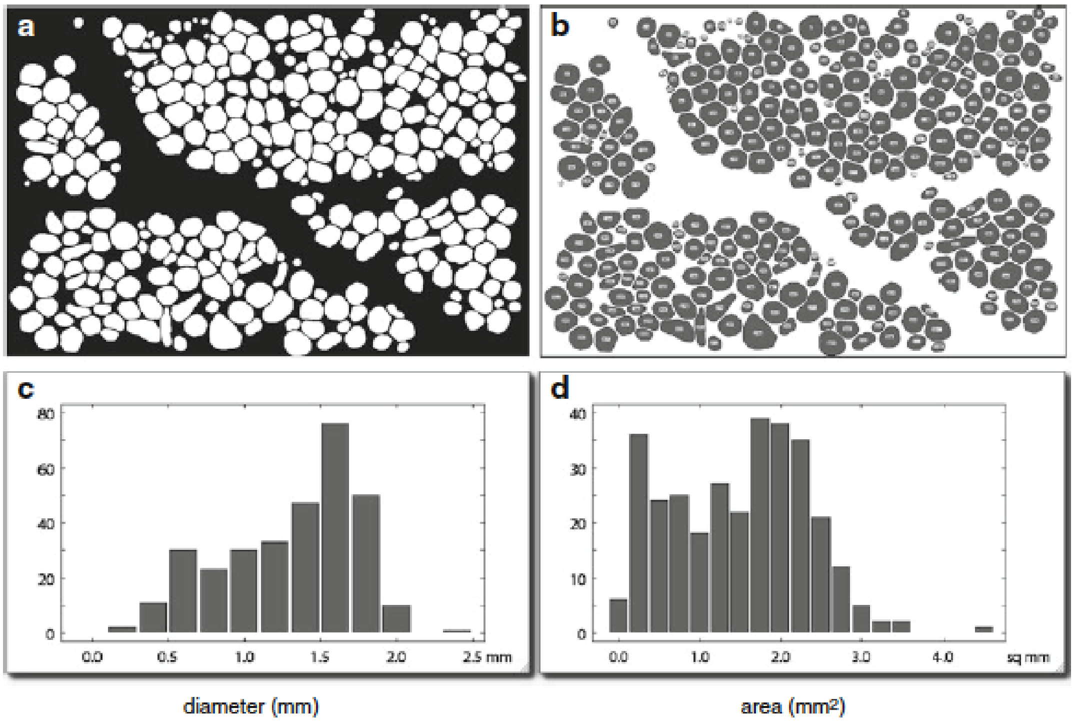


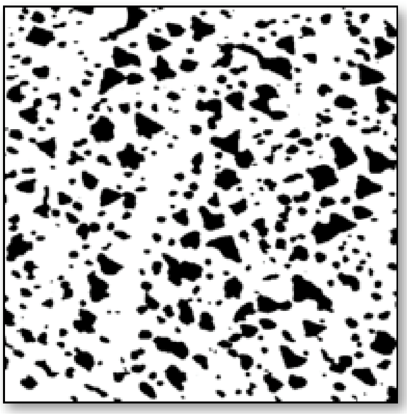
Image Measurements

Grain Size -Example

Oolithic limestone sample from the Jura mountains in Switzerland –
ImageJ -> Analyse particles/Distribution



Particle Analysis (example in Fiji/ImageJ)

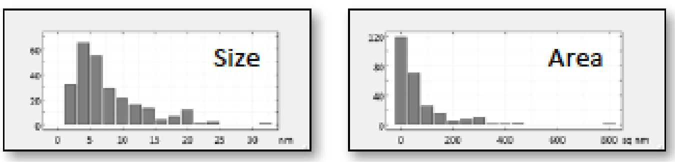


→

	Area	Mean	StDv	X	Y	Len	Majr	Minr	Angle	Min	Max
1.	324.38	0.74	0.07	109.15	329.47	105.67	34.59	11.94	43.0	0.59	0.91
2.	6.86	0.63	0.02	135.40	341.15	11.54	4.65	1.88	9.0	0.59	0.65
3.	5.39	0.50	0.01	294.51	341.03	8.74	3.33	2.06	177.5	0.59	0.60
4.	4.90	0.61	0.02	340.76	339.57	12.36	5.79	1.08	94.9	0.59	0.65
5.	1.47	0.59	0.00	43.40	340.90	4.37	2.37	0.79	0.0	0.59	0.59
6.	10.29	0.62	0.03	129.07	339.20	11.13	4.40	2.98	172.8	0.59	0.67
7.	24.50	0.67	0.05	298.94	335.79	17.89	6.72	4.64	170.9	0.59	0.77
8.	5.88	0.65	0.04	172.26	336.17	8.74	3.63	2.06	175.2	0.59	0.70
9.	48.02	0.70	0.08	312.58	332.72	27.79	9.18	6.66	54.0	0.59	0.87
10.	40.67	0.64	0.03	28.80	332.96	24.07	8.05	6.43	5.1	0.59	0.68
11.	34.30	0.73	0.09	279.41	332.26	21.44	7.83	5.57	20.0	0.59	0.89
12.	6.37	0.61	0.01	252.92	333.85	9.73	4.17	1.95	13.0	0.59	0.63
13.	1.96	0.60	0.01	14.70	333.02	4.95	1.91	1.30	0.0	0.59	0.61
14.	5.39	0.63	0.03	264.09	331.23	8.33	3.33	2.06	2.5	0.59	0.67
15.	21.56	0.65	0.04	298.71	329.05	16.08	5.95	4.61	149.8	0.59	0.73
16.	5.39	0.60	0.01	213.04	329.57	8.53	3.49	1.97	11.3	0.59	0.62
17.	96.04	0.73	0.12	315.38	321.78	46.50	15.97	7.46	118.2	0.59	1.00
18.	44.59	0.65	0.03	223.54	326.75	24.82	9.36	6.06	175.6	0.59	0.70
19.	19.60	0.61	0.02	144.71	325.83	18.88	7.72	3.23	169.3	0.59	0.64
...	...	...	...	...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...	...	...	...

↙

Mean nearest neighbour distance = 13 ± 5 nm
Nearest neighbour lies in azimuthal direction 83° (anisotropy = 0.19)



Estimating Size

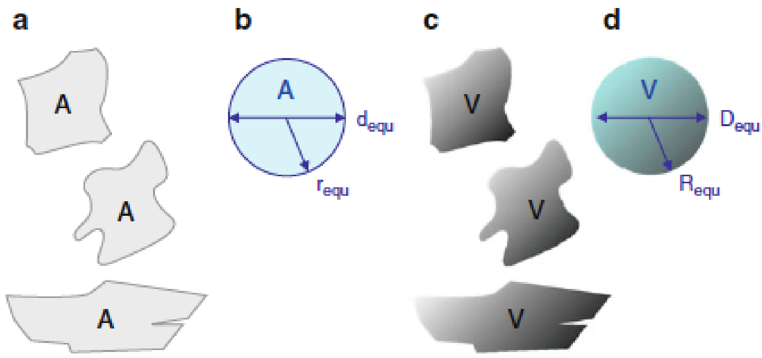
mating area

nting pixels: The area of a particle A is given by the number of pixels in that phase and the area density is obtained l
ting the number of pixels of the particles to the total number of pixels -> Measure command in ImageJ

mating volume Vα of phase α
›rresponds to the area fraction

$$V_\alpha = \int A_\alpha(z)\, dz$$

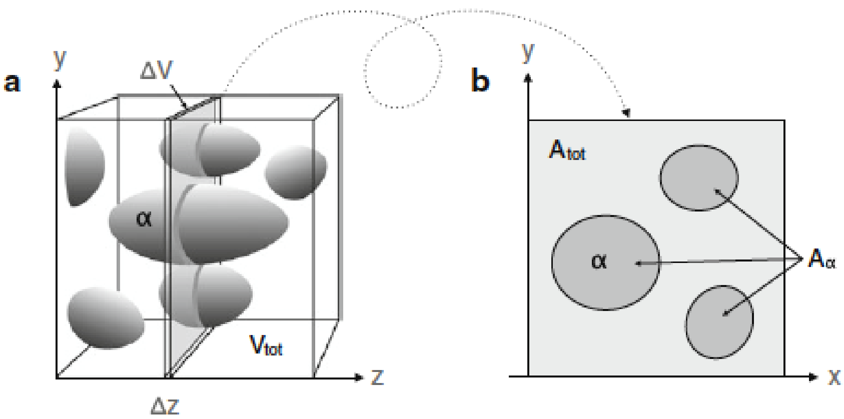
$$\frac{\bar{A}_\alpha}{A_{tot}} = \frac{V_\alpha}{V_{tot}}$$



The best estimate is by using the cross-sectional area and the concept of area-equivalent circle/eclipse

Particle Analysis – How it works

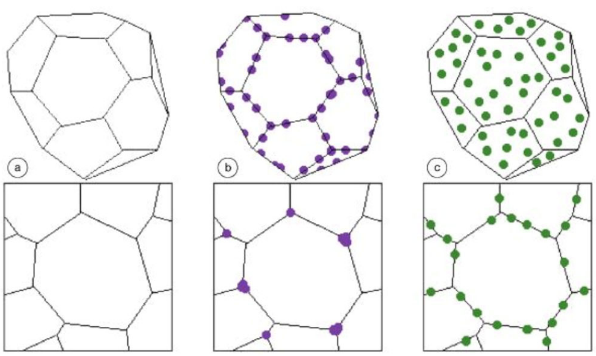
In 3D



2D Images

In 3D

In 2D



Software Practice: ImageJ [or Fiji]

IMAGEJ

An open platform for scientific image analysis

http://imagej.net/ImageJ2

https://imagej.net/Fiji/Downloads