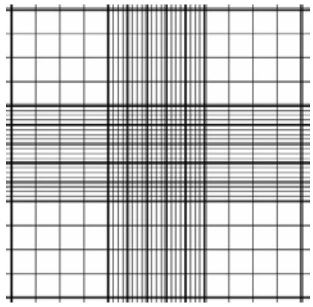
Determination of the Pixel Size

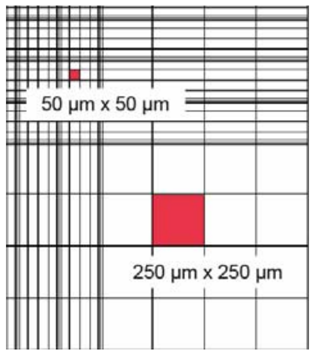
1. General Information

This application note describes how to measure and calculate the correct pixel size of microscopic images. Pixel size calibration is necessary for image processing such as tracking, scale bars or other image analysis software, like ibidi Chemotaxis and Migration Tool. The basic assumption is that all pixels are squares and there is no image distortion in any direction. We use [µm/pixel] instead of [pixel/µm] because pixels are considered as whole-numbered so the pixel size is the edge length of one pixel in µm.



<u>Material needed:</u>

- Cell counting chamber "Neubauer improved"
- Microscope with digital camera
- Computer with graphics software



The "Neubauer improved" cell counting chamber consists of two different kinds of non-subdivided squares suitable for definition of microscopic length.

Large squares: 250 µm edge length
Small squares: 50 µm edge length

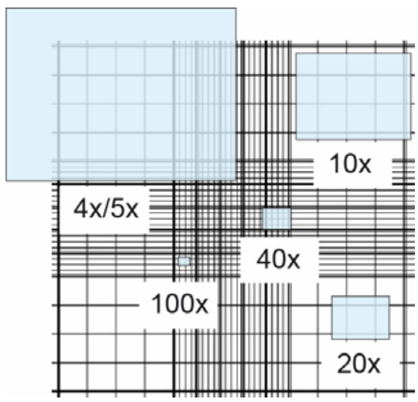
2. Preparation of the material

- Put the cell counting chamber on your microscope stage. Neither mount a coverslip nor fill the chamber with water or cell suspension. Face the gridded side towards the objective lens.
- When using oil immersion objectives use appropriate immersion oil and mount an appropriate coverslip according to the manufacturer's recommendations. Fill the counting chamber with water.
- Adjust the counting chamber in a way that all lines appear absolutely parallel with your microscope camera.

3. Image acquisition

- Take an image with your camera setup. Use the areas recommended for the objective lens you use.

Objective lens	**Squares to be measured (edge length)**
4x/5x	2 Large squares (= 2 x 250 µm)
10x	2 Large squares (= 2 x 250 µm)
20x	1 Large square (= 250 µm)
40x	2 Small squares (= 2 x 50 µm)
100x	1 Small square (= 50 µm)



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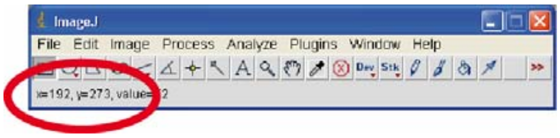
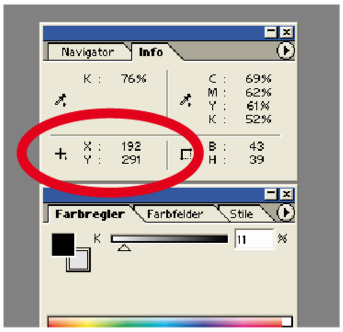
4. General image processing

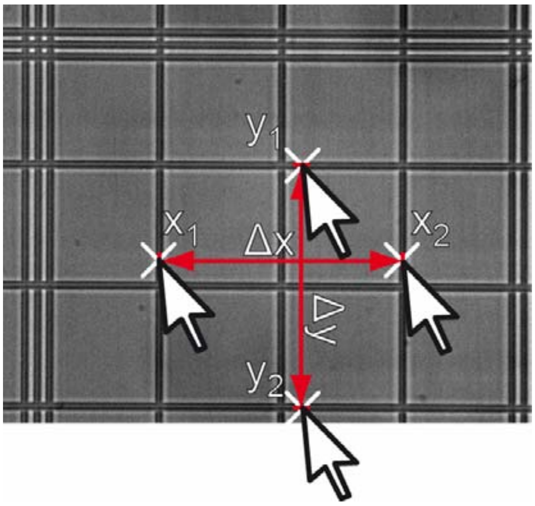
- Open image with graphics software such as Adobe Photoshop, ImageJ, IrfanView or similar software.
- Move cursor to the measuring position in the image and read the x and y positions.
- Calculate Δx and Δy by subtraction.
- Optionally, make a rectangle or line selection and read the length of the line in pixels.
- Find out in which area of the Neubauer chamber you took the image and how many squares you measured.
- Calculate the pixel size in x and y direction using the general formulas. Keep in mind that the pixel size depends on objective lens and camera settings like binning and image resolution.



Example ImageJ:
x and y positions



Example Adobe Photoshop:
x and y positions



Example 40x lens – two squares of 50 µm edge length are measured

General formulas for Pixel Sizes P in x and y direction:

$$P_x \left[\frac{\mu m}{px}\right] = \frac{\text{Real distance in x direction } [\mu m]}{\text{Distance in pixels} = \Delta x = x_2 - x_1 \text{ [px]}}$$

$$P_y \left[\frac{\mu m}{px}\right] = \frac{\text{Real distance in y direction } [\mu m]}{\text{Distance in pixels} = \Delta y = y_2 - y_1 \text{ [px]}}$$

- Ideally, both values P_x and P_y are identical or with very small difference only.
- In case P_x and P_y differ strongly, contact your microscope and/or camera manufacturer and report image distortion.

For identical pixel sizes in x- and y-direction:

$$P_x = P_y$$

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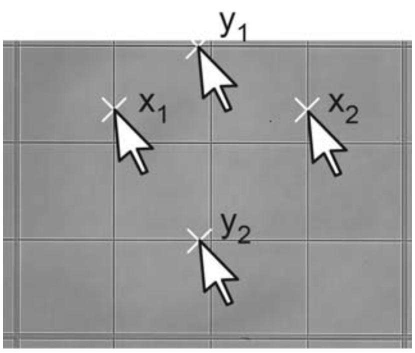
5. Example image processing

10x objective lens



ibidi Example	Your Data
x_1: 368 px	x_1: _ _ _ px
x_2: 1013 px	x_2: _ _ _ px
Δx: 645 px	Δx: _ _ _ px

$$P_x \left[\frac{\mu m}{px}\right] = \frac{2 \cdot 250\ \mu m}{645\ px} \qquad P_x = 0.775\,\frac{\mu m}{px}$$

$$P_x \left[\frac{\mu m}{px}\right] = \frac{2 \cdot 250\ \mu m}{\_\,\_\,\_\ px} \qquad P_x = \_.\_\,\_\,\_\,\frac{\mu m}{px}$$

ibidi Example	Your Data
y_1: 18 px	y_1: _ _ _ px
y_2: 662 px	y_2: _ _ _ px
Δy: 644 px	Δy: _ _ _ px

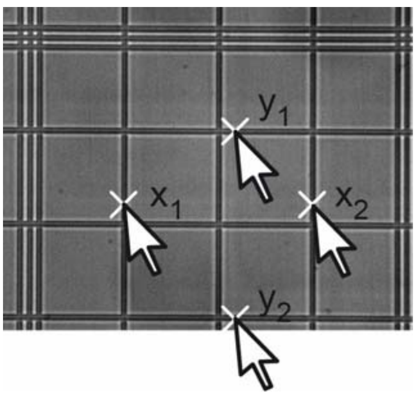
$$P_y \left[\frac{\mu m}{px}\right] = \frac{2 \cdot 250\ \mu m}{644\ px} \qquad P_y = 0.776\,\frac{\mu m}{px}$$

$$P_y \left[\frac{\mu m}{px}\right] = \frac{2 \cdot 250\ \mu m}{\_\,\_\,\_\ px} \qquad P_y = \_.\_\,\_\,\_\,\frac{\mu m}{px}$$

$$P_x = P_y = 0.78\,\frac{\mu m}{px}$$

$$P_x = P_y = \_.\_\,\_\,\frac{\mu m}{px}$$

40x objective lens



ibidi Example	Your Data
x_1: 192 px	x_1: _ _ _ px
x_2: 492 px	x_2: _ _ _ px
Δx: 300 px	Δx: _ _ _ px

$$P_x \left[\frac{\mu m}{px}\right] = \frac{2 \cdot 50\ \mu m}{300\ px} \qquad P_x = 0.333\,\frac{\mu m}{px}$$

$$P_x \left[\frac{\mu m}{px}\right] = \frac{2 \cdot 50\ \mu m}{\_\,\_\,\_\ px} \qquad P_x = \_.\_\,\_\,\_\,\frac{\mu m}{px}$$

ibidi Example	Your Data
y_1: 197 px	y_1: _ _ _ px
y_2: 495 px	y_2: _ _ _ px
Δy: 298 px	Δy: _ _ _ px

$$P_y \left[\frac{\mu m}{px}\right] = \frac{2 \cdot 50\ \mu m}{298\ px} \qquad P_y = 0.336\,\frac{\mu m}{px}$$

$$P_y \left[\frac{\mu m}{px}\right] = \frac{2 \cdot 50\ \mu m}{\_\,\_\,\_\ px} \qquad P_y = \_.\_\,\_\,\_\,\frac{\mu m}{px}$$

$$P_x = P_y = 0.33\,\frac{\mu m}{px}$$

$$P_x = P_y = \_.\_\,\_\,\frac{\mu m}{px}$$

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