Determination of the Pixel Size of Microscopy Images

"Pixel size" refers to the physical dimensions of a single pixel in a microscopic image, typically measured in micrometers (µm). In microscopy, the pixel size helps determine the field of view and the level of detail in an image, as well as how the image scale corresponds to real-world measurements. Please note that in this Application Note, "pixel size" does not refer to the physical dimensions each pixel occupies on a camera sensor.

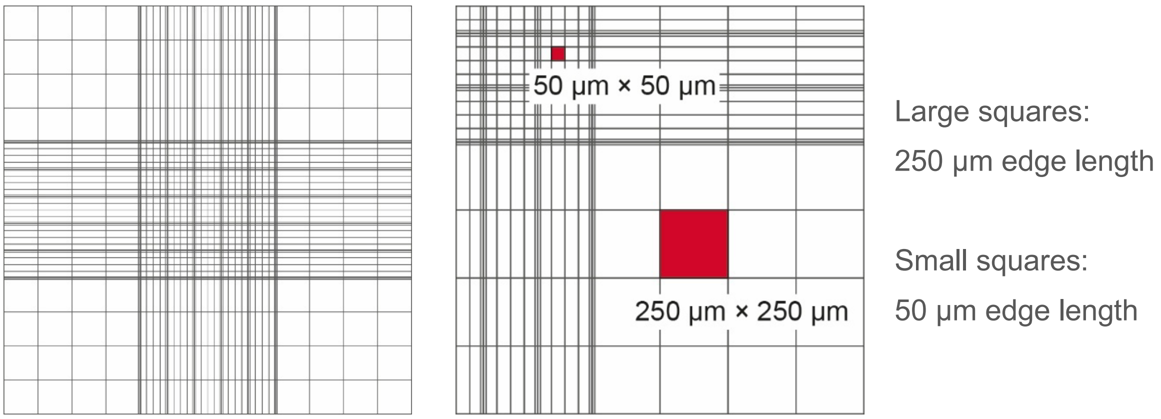
This Application Note explains how to measure and calculate the appropriate pixel size of microscopic images. Calibrating the pixel size is essential for image processing tasks such as tracking, creating scale bars, or using image analysis software such as the ibidi Chemotaxis and Migration Tool.

To calculate the pixel size, we assume that all pixels are square and that there is no image distortion in any direction. In this Application Note, we will use the unit [µm/pixels] instead of [pixels/µm]. If you need pixels/µm, calculate the reciprocal by using 1/result.

1 Material

Note: Special rulers and calibration slides are available to calibrate and determine the pixel size. However, one simple method is using a “Neubauer improved” cell counting chamber.

- Cell counting chamber “Neubauer improved”, optionally immersion oil and coverslip
- Microscope with a digital camera
- Computer with graphics software (e.g., Adobe Photoshop, ImageJ, IrfanView)



The “Neubauer improved” cell counting chamber grid contains two different kinds of non-subdivided squares, which are suitable for defining the microscopic length.

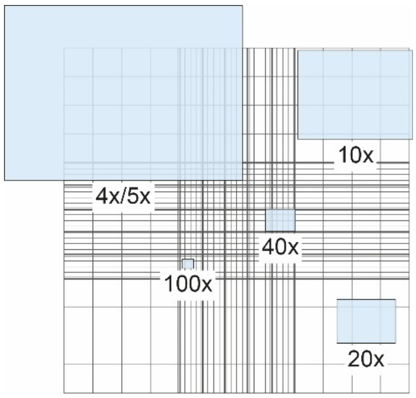
2 Material Preparation

- **When using air objectives**, place the blank cell counting chamber on your microscope stage. Do not mount any coverslip or fill the chamber with any water or cell suspension. Ensure the gridded side faces the objective lens.
- **When using oil immersion objectives**, apply the appropriate immersion oil to the chamber and mount an appropriate coverslip according to the manufacturer's recommendations. Fill the counting chamber with water.
- In live mode, align the counting chamber so its grid lines are parallel with the top and left screen edges. Adjust slightly until lines are perfectly parallel.

3 Image Acquisition

Using your camera setup, take an image of the recommended areas for the respective objective lens as shown in the figure below.

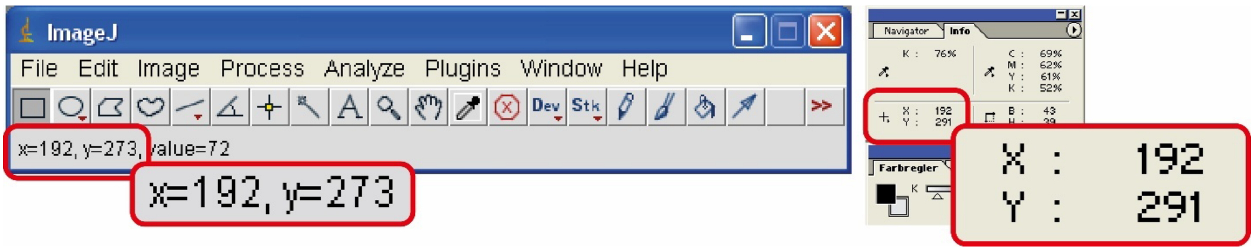
Objective lens	Squares to be measured (edge length)
4x/5x	2 Large squares (= 2 × 250 µm)
10x	2 Large squares (= 2 × 250 µm)
20x	1 Large square (= 250 µm)
40x	2 Small squares (= 2 × 50 µm)
100x	1 Small square (= 50 µm)



4 General Image Processing

- Open the image with graphics software such as Adobe Photoshop, ImageJ, IrfanView, or similar software.
- Move the cursor to one edge of a square in the image and note the x and y positions.
- Move the cursor to the opposite edge of one or multiple squares and again note the x and y positions.

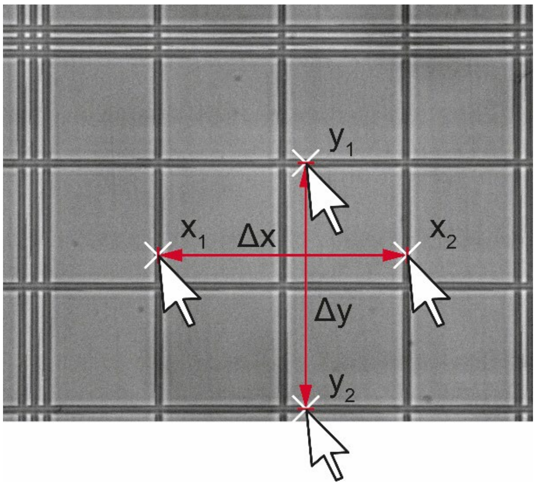
Note: The number of squares is not relevant; only the total size of one or more squares must be a known variable.



Example x and y positions using ImageJ (left) and Adobe Photoshop (right).

- Calculate Δx and Δy by subtracting the x and y values.

  Alternatively: Instead of using single x and y values by moving the cursor, consider making a rectangular or line selection to measure the length of the line in pixels.



Example using a 40x lens: four squares of 50 µm edge length are measured.

- To calculate the pixel size P in the x and y directions, divide the length of the analyzed edge (in micrometers, µm) by the number of pixels (Δx and Δy) using these general formulas:

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

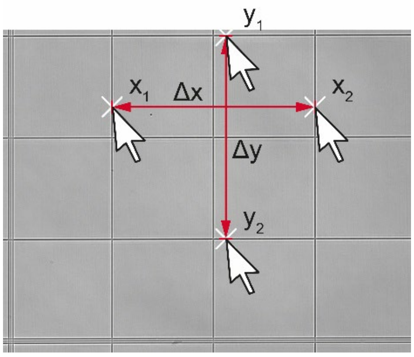
$$P_y\left[\frac{\mu m}{px}\right] = \frac{\text{Real distance in y direction } [\mu m]}{\text{Distance in pixels} = \Delta y = y_2 - y_1 \, [px]}$$

- The edge length (real distance in x or y direction) in micrometers will depend on the area imaged in the Neubauer chamber, which may vary between 50 µm and 250 µm.
- For squared pixels, P_x and P_y should be identical or have only a minimal difference. In case P_x and P_y differ strongly, contact your microscope and/or camera manufacturer and report image distortion.

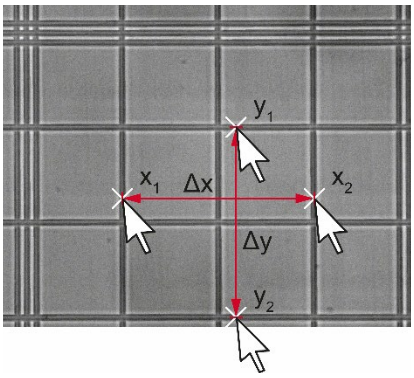
Note: Keep in mind that the calculated pixel size depends on the objective lens and camera settings, such as binning and image resolution.

5 Example Image Processing

10x Objective Lens



	ibidi Example	Your Data
x_1:	368 px	_ _ _ px
x_2:	1013 px	_ _ _ px
Δx:	645 px	_ _ _ px
	$P_x\ [\frac{\mu m}{px}] = \frac{2 \cdot 250\ \mu m}{645\ px}$	$P_x\ [\frac{\mu m}{px}] = \frac{2 \cdot 250\ \mu m}{\_\ \_\ \_\ px}$
	$P_x = 0.775 \frac{\mu m}{px}$	$P_x = \_ . \_\ \_\ \_ \frac{\mu m}{px}$
y_1:	18 px	_ _ _ px
y_2:	662 px	_ _ _ px
Δy:	644 px	_ _ _ px
	$P_y\ [\frac{\mu m}{px}] = \frac{2 \cdot 250\ \mu m}{644\ px}$	$P_y\ [\frac{\mu m}{px}] = \frac{2 \cdot 250\ \mu m}{\_\ \_\ \_\ px}$
	$P_y = 0.776 \frac{\mu m}{px}$	$P_y = \_ . \_\ \_\ \_ \frac{\mu m}{px}$
	$P_x = P_y = 0.78 \frac{\mu m}{px}$	$P_x = P_y = \_ . \_\ \_ \frac{\mu m}{px}$

40x Objective Lens	ibidi Example	Your Data
	x_1: 192 px x_2: 492 px Δx: 300 px	x_1: _ _ _ px x_2: _ _ _ px Δx: _ _ _ px
	$P_x\ [\frac{\mu m}{px}] = \frac{2 \cdot 50\ \mu m}{300\ px}$ $P_x = 0.333 \frac{\mu m}{px}$	$P_x\ [\frac{\mu m}{px}] = \frac{2 \cdot 50\ \mu m}{\_\ \_\ \_\ px}$ $P_x = \_.\_\ \_\ \_ \frac{\mu m}{px}$
	y_1: 197 px y_2: 495 px Δy: 298 px	y_1: _ _ _ px y_2: _ _ _ px Δy: _ _ _ px
	$P_y\ [\frac{\mu m}{px}] = \frac{2 \cdot 50\ \mu m}{298\ px}$ $P_y = 0.336 \frac{\mu m}{px}$	$P_y\ [\frac{\mu m}{px}] = \frac{2 \cdot 50\ \mu m}{\_\ \_\ \_\ px}$ $P_y = \_.\_\ \_\ \_ \frac{\mu m}{px}$
	$P_x = P_y = 0.33 \frac{\mu m}{px}$	$P_x = P_y = \_.\_\ \_ \frac{\mu m}{px}$