Phase-Contrast & Fluorescence Microscopy of Adherent Cells grown on ECIS Electrodes

Prof. Joachim Wegener
University of Regensburg
Institut für Analytische Chemie,
Chemo- und Biosensorik
93040 Regensburg
Germany

Content

1 General Remarks

When cells are grown on ECIS™ culture ware, they can be examined by both phase contrast and fluorescence microscopy in the wells of the ECIS™ arrays, even though they are attached to a gold-film electrode. Compared to ordinary cell culture substrates such as treated plastic culture dishes or cover glasses, there are some peculiarities arising from both the gold film substrates and the chambers that are glued to the ECIS™ base slide by a silicone adhesive.

In general, the most convenient means to obtain good cell images involve using an *inverted microscope* (Fig. 1) set up for *phase contrast microscopy*. When the cells are to be studied by *fluorescence contrast,* an *upright microscope* (Fig. 2) is more convenient and the procedures are much simpler.

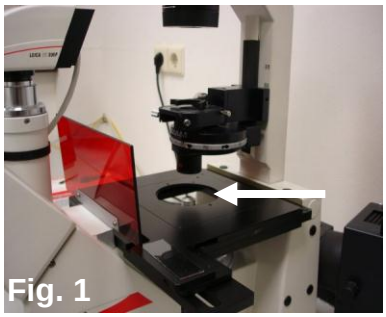
Fig. 1

The only principle difference between both types of microscopes is the position of the objectives that are either underneath the sample stage in *inverted microscopes* (white arrow in Fig.1) or above the stage in *upright microscopes* (white arrow in Fig.2). The different positions of the objectives provide many practical differences that affect sample handling but the optical performance is not different in principle.

If only one type of microscope is available, there are still workarounds to perform both *phase contrast* and *fluorescence contrast* with either kind of instrument. However, as is discussed later in this tutorial, the procedures are less convenient and also the image quality may be compromised.


Fig. 2

2 Phase Contrast Microscopy

2.1 Inverted Microscope

Using an inverted microscope for phase contrast microscopy of cells adhered to ECIS™ electrodes is very straightforward and not different from conventional cell culture substrates.

You can study the cells with the ordinary microscope settings either when the array is still in the protecting Petri dish (Fig. 3) or you can put the array directly on the stage of the microscope (Fig. 4).


Fig. 3

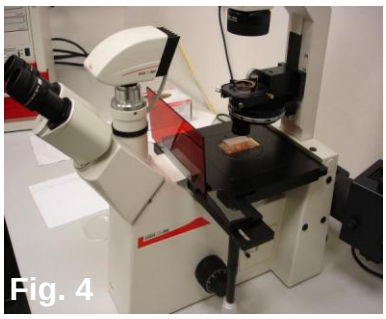
Fig. 4

The gold-film of the electrodes is ~ 100 nm thick. It is transparent but certainly absorbs some of the transmitted light. Thus, you may find that the light source needs to be adjusted to higher intensities to get a good view of cell upon the electrode (Fig. 5). For quick and routine checking on the status of the cells, this is about all that needs to be considered.

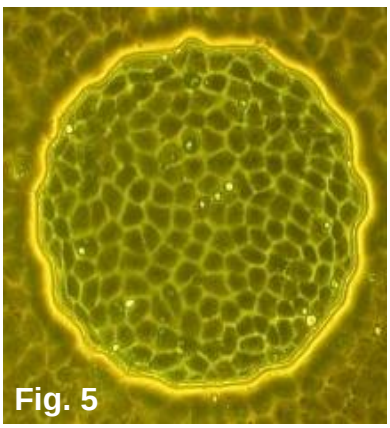
Fig. 5

For high quality images you are probably used to check for appropriate Köhler illumination and to adjust the Bertrand lens for a given sample. Using cells on ECIS™ electrodes, you may find that each well of an electrode array requires individual adjustment of the Bertrand lens (Fig. 6) to get the best images. We haven‘t studied the reason for this, but it is likely due to different reflections at the gold surface and different light scattering at the liquid / air interface for each well, as the electrode is not always centered perfectly under the liqiud meniscus.

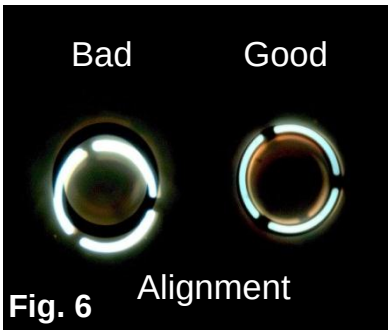

Fig. 6

2.2 Upright Microscope

When an upright microscope is used, the height of the 8 well chamber requires use of a long-distance objective that is typically not included in a microscope package. With a normal objective the working distance *d* (see Fig. 7) is commonly less than 3 mm. With such an objective, the cells cannot be brought into focus on the bottom of the well in an *upright microscope,* as the 8well chamber is app. 12 mm in height.

You can find the working distance of your objective typed with other parameters on the objective body. The example in figure 7 (arrrow) indicates a working distance of **1 mm** which is very typical for an objective with this resolution.

Fig. 7

If a long-distance objective is not available, you have to remove the 8-well chamber top to do microscopy with an upright microscope. This will be discussed later in this tutorial (page 6).

3 Fluorescence Microscopy

General

The gold-film electrodes are more problematic for *fluorescence* than for *phase contrast microscopy*. In addition, it is quite a bit easier to use an *upright microscope* than an *inverted* one for *fluorescence contrast*. The reason is as follows: in fluorescence microscopy, it is the low intensity fluorescent light emitted from the sample that is used for imaging. This weak fluorescence light is not transmitted effectively through the gold film, as it is absorbed and/or reflected by the gold-film. As a consequence, the objective should be on the side of the gold electrode and not beneath it. Thus, it is important to use an *upright microscope* (see Fig. 2) and not an *inverted microscope* as shown in figure 8. The only way to make use of an *inverted microscope* for *fluorescence contrast* involves removing the 8 well chamber top, and flipping the ECIS™ slide upside down, putting the objective lens on the same side as the gold electrodes. This will be described below under the section *Inverted Microscope*. We will start here with the procedures applicable for *upright* instruments.

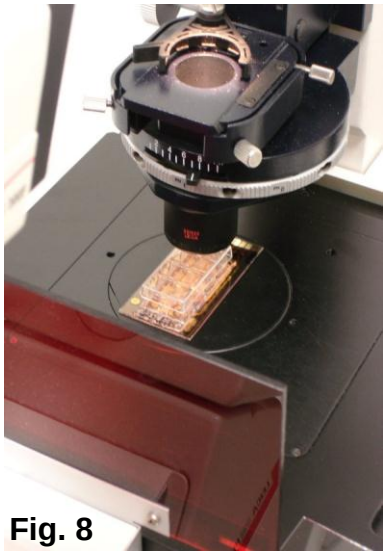

Fig. 8

Upright Microscope

The staining of the cells can be conveniently performed in the ECIS™ wells including chemical fixation if needed (Fig. 9). Please keep in mind that the insulating film on the bottom of the wells is base-sensitive and not tolerant of most organic solvents. Thus, staining protocols that require fixation with solvents like methanol, ethanol or acetone are not compatible with the ECIS™ culture ware and have to be replaced by protocols based upon aldehyde fixation and subsequent membrane permeabilization with small amounts of detergents.

Fig. 9

3 Fluorescence Microscopy

After all the staining and washing steps have been performed, the 8 well chamber top has to be removed unless a *long-distance objective* is available. With a long-distance objective the sample can be directly studied in *fluorescence contrast*. However, even if available, long distance objectives are usually not made for high-resolution imaging. Thus, the following procedure must usually be followed if you intend to make detailed studies of the cells.

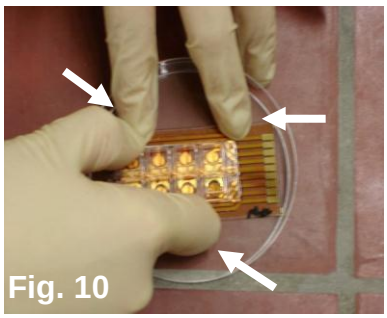
Fig. 10

To remove the 8 well chamber from the base slide, the liquid in the wells should first be aspirated leaving only a thin liquid film on the cells to prevent them from drying.

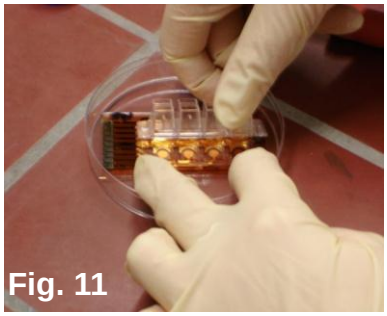
Fig. 11

When lifting off the top well assembly, be careful not to inadvertently contact the cell layer. We found that it is helpful to press the slide to a plane surface (fig. 10) along both long sides (arrows in Fig. 10) if possible. Then carefully tilt the chamber top to the side as shown in figures 11 and 12.

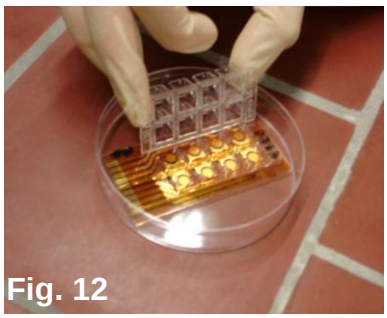
Fig. 12

Sometimes upon removing the wells, the upper chambers and base slide remain connected by filaments of the silicon adhesive. These can be easily cut using a pair of scissors (Fig. 13).


Fig. 13

3 Fluorescence Microscopy

The ECIS™ culture ware base slide is then placed on the bottom of a Petri dish or any other appropriate vessel and weighted down by pieces of plastic (Fig.14) to immobilize it.

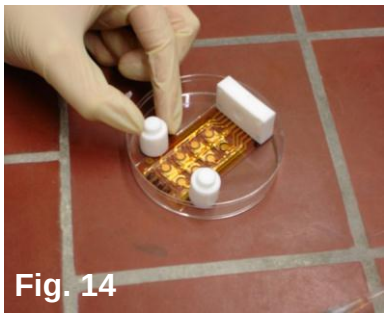
Fig. 14

Carefully add back buffer to the samples. The leftovers of the silicone seal form a bit of a chamber that will hold a small volume of fluid over the electrode (Fig. 15).

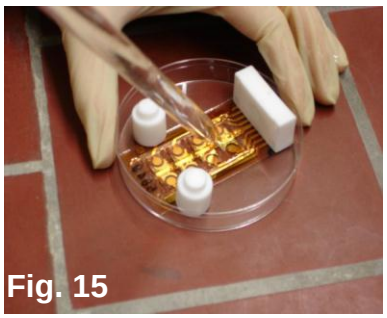
Fig. 15

The subsequent procedures depend on whether a *water immersion objective* is available or not. Water immersion objectives can be dipped into the buffer above the sample and employ the buffer as immersion fluid. They can be easily recognized by their water tight seal at the front lens (blue arrow in fig. 16) as well as the letter „W“ on the description panel of the objective (red arrow in fig. 16).

Fig. 16

Water Immersion Objective Available

When you have access to a *water immersion objective,* the whole Petri dish is flooded carefully with buffer (fig. 17). The plastic weights keep the base slide from buoying upwards. Introducing the liquid should be done gradually and with care, as it can impose a mechanical stress to the cells.


Fig. 17

3 Fluorescence Microscopy

Water Immersion Objective Available (cont.)

The Petri dish with the ECIS™ slide fixed to the bottom can then be mounted on the stage of the microscope with the objective dipping into the fluid from above (Fig.18).

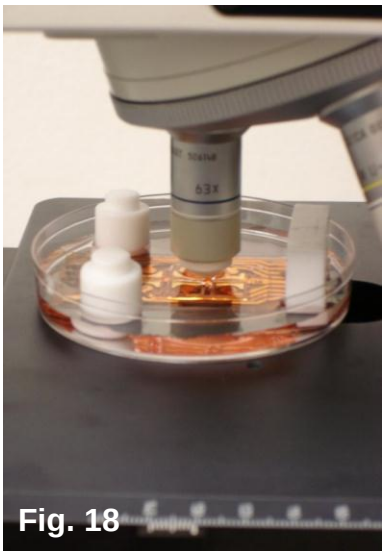
Fig. 18

With this setup it is possible to perform high resolution images from fluorescently stained cells on the electrode surface. Please consider the statements regarding selection of fluorophores for staining in combination with ECIS™ culture ware at the end of this tutorial.

As an example, figure 19 shows a layer of NRK cells after applying an elevated electric field for cell wounding. The cell layer was then stained by a combination of two fluorescent dyes: Calcein-AM provides living cells with a green cytoplasmic fluorescence, while Ethidium Homodimer stains the nuclei of dead cells red. The image has been recorded with a water immersion objective (NA = 0.9).

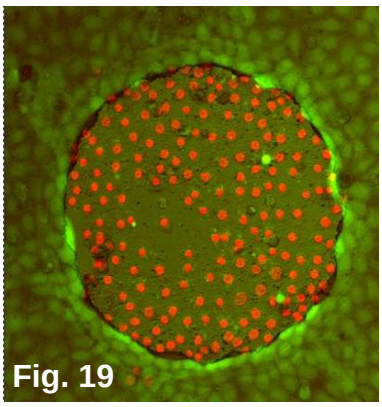
Fig. 19

Water Immersion Objective <u>Not</u> Available

When a *water immersion objective* is not available, the 8 well chamber top is removed as described above. A thin film of liquid should remain on the slide to cover the cells and prevent them from drying. These slides can now be used directly with “dry“ objectives (no immersion fluid) as long as the liquid film is thin enough to bring the objective in working distance without touching the liquid. Samples prepared in this manner will have to be examined rather fast, as the liquid will evaporate and dry out the cells.

Water Immersion Objective Not Available (cont.)

Alternatively, you can mount a glass coverslip over the sample leaving a small channel filled with buffer above the cells. The plane of the coverslip may not be perfectly parallel to the stage, and thus you may experience different focus levels as you move across the specimen. This is not an ideal procedure but provides much more time before the sample dries out. In addition to this protection from fluid evaporation, the extra coverslip allows one to use oil immersion objectives.

Inverted Microscope

With an *inverted microscope* the procedures are even less convenient. The eight-well chamber has to be removed and a coverslip must be mounted over the sample. Finally the ECIS™ base slide with the coverslip is flipped over to lie upside down on the microscope stage. The objective is then approached from below. This clearly is not the most desirable setup but serves as a workaround if an upright microscope is not available.

3 Fluorescence Microscopy

Compatibility of Fluorophores

During production of the ECIS™ culture ware the gold film on the base-slide is coated with an insulating polymer. This photoresist film is then removed in defined areas by means of photolithography providing the µm-sized electrodes. Figure 20 shows a scanning electron micrograph of a 250 µm diameter electrode.

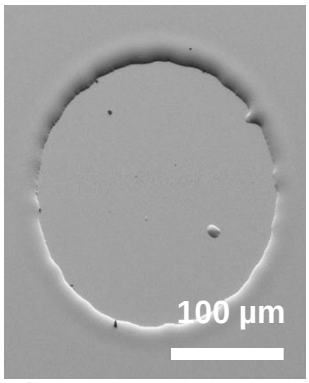

Fig. 20

As a result of this production method, there is always insulating polymer around the small gold electrode. Like most other organic polymers this photoresist exhibits *auto-fluorescence* dependent upon the wavelength of the incident light. In addition the gold film has some amazing optical properties, which may lead to auto-fluorescence or fluorescence quenching.If you have a choice of fluorophores you may want to consider this behavior of the ECIS™ cultureware.

Figure 21 shows four fluorescence micrographs of an ECIS™ gold electrode being exposed to excitation light of 340-380 nm as used to excite the nuclear stain DAPI emitting blue fluorescence. The exposure time was gradually increased from 1s to 4s.

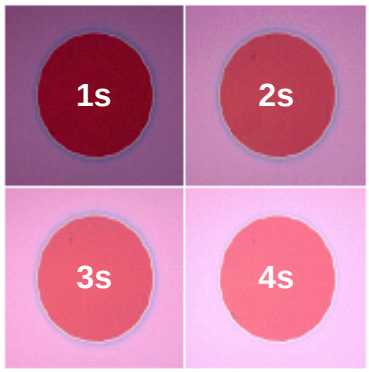

Fig. 21

Figure 22 shows the same collection of images for excitation light of 480-500 nm as used to excite FITC which emits green fluorescence.

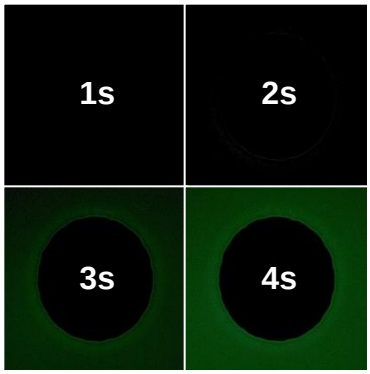

Fig. 22

Figure 23 shows the corresponding images for an excitation light of 540-570 nm as used for TRITC, known to emit red fluorescence.

It depends upon the choice of fluorophore for staining and the exposure time whether the autofluorescence of the ECIS™ cultureware will interfere with microscopy of your sample. For FITC/TRITC labeled samples, image quality is only affected - if at all - when those cells adhering to the photoresist will be included in the image. If possible, staining with dyes absorbing blue light is preferable (Fig. 22).

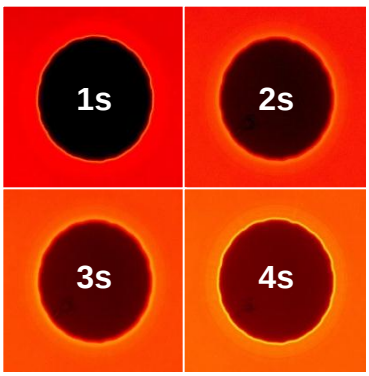

Fig. 23