



**RingAssay a program for evaluating cell mobility**

**C. Karreman 2015 - 2016**

**As published in:**

Nyffeler J., Karreman C., Leisner H., Kim Y. J., Lee G., Waldmann T., and Leist M. (2016), Design of a high-throughput human neural crest cell migration assay to indicate potential developmental toxicants. *Altex* **34**, p75-94. - doi: 10.14573/altex.1605031.



## Index

<b>Installing the Ringassay Program</b>	4
<b>A few words of warning!</b>	
What we use	
<b>Running the RingAssay program</b>	5
Loading the photos	
<b>Options available after loading</b>	
The “Overview” button	6
The “Go” button	
<b>Selecting / deselecting wells</b>	7
<b>Showing the data for a single well and     setting the parameters for analyzing.</b>	
The tabsheet labelled:”Raw Two Channel”	
The “Apply” button	8
The “Save” button	
The tabsheet labelled: “Blue channel”	
The tabsheet labelled: “Green channel”	
The tabsheet labelled: “Immigrant Cells”	9
The panel called ““Results”	10
The number of nuclei	
The number of pixels	
The number of calculated nuclei	
Average distance	
Total number of nuclei	
The tabsheet labelled: “Cells”	11
The panel called “Shape and Orientation”	13





## Installing the Ringassay Program

The Ringassay program is a 32bit program for Windows. It will not run naturally on Mac or Linux. If you have Wine installed there is a very good chance it will run on the latter two OSes. However I have **NOT** checked this.

There is no need for any registration or special rights for you to install and use the program. You just copy the Ringassay.exe file from the Ringassay.zip file onto your hard disk and it will run. Alternatively you can- of course- use the “extract” function to do this.

### A few words of warning!



This program was custom made just for solving the problems of measuring an enormous amount of cell related data in our laboratory. It was programmed for using the formats we use, on our computer setup and with our aims in mind. So it is not as flexible as it could have been.

#### What we use:

We use the 96-well format exclusively in all our ring assays.

To analyze these we use H-33342 (blue channel) and Calcein-AM (green channel)

For the evaluation of the plates we use the automated microscope system of Cellomics (Thermo Fischer), the Arrayscan VTI.

The results are exported as 8 pictures per well (four pictures will cover the stopper area and this for the two channels used). Pictures are in TIFF format and dimensions are 512 x 512 pixels. The default naming of the Cellomics software is used. E.g. ARRAYSCANVTI\_151212140001\_B02f00d1, indicating well (B02), position of photo (f0= bottom left) and channel (d1 = green). The RingAssay program uses only this part of the name to determine whether a photo is present. Should you use the program to evaluate photos from an other source, you should name your pictures similarly.

All pictures of a plate must be saved in one folder. Not all wells have to be used.

The output can be directed directly to Microsoft Excel - this program has to be installed on the computer in order to use this feature.

## Running the RingAssay program

### Loading the photos

As soon as you start the program a new window will appear that will cover the whole of your screen. The control panel (see Fig. 1) will be shown. To start evaluating your photos go to the menu and select "Open Pictures". Go to your folder containing the photos and click on any one of them. There is another entry for loading "Settings", for this see page 6.

The RingAssay program will now check which of the wells was photographed and whether all photos are present. It will indicate this schematically in the upperpart of the control panel, see Fig. 1B.

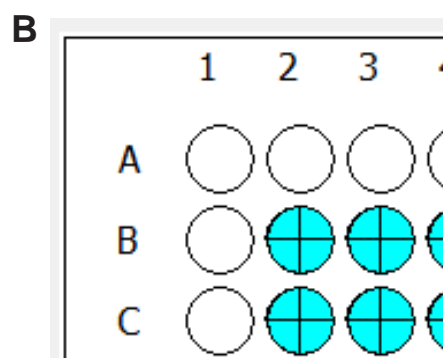
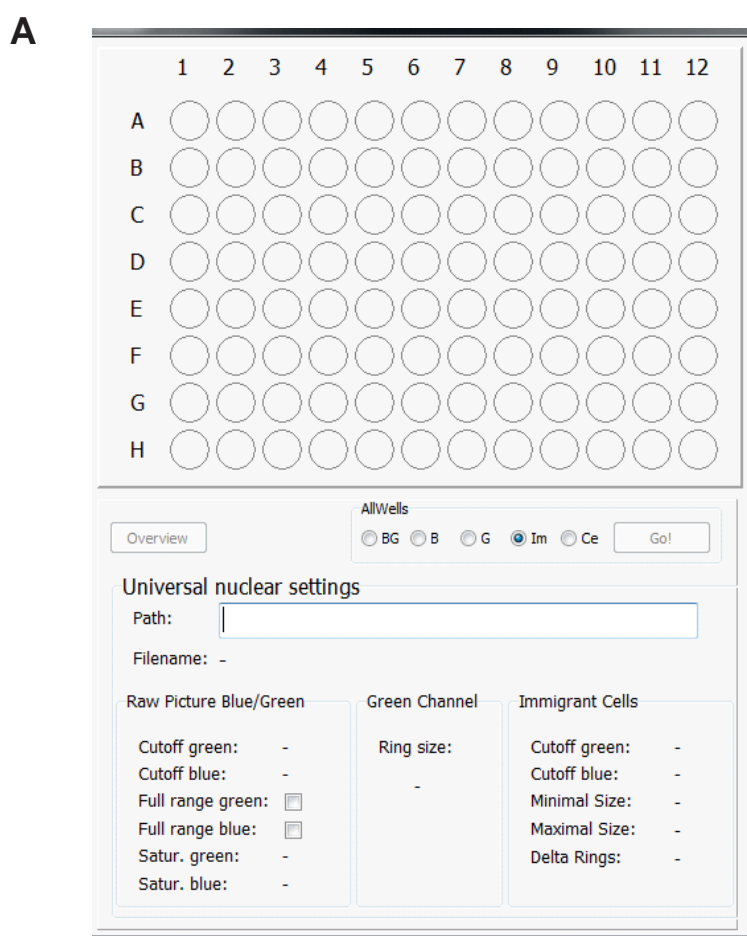


Fig. 1 The Control Panel

- A) If you open the RA program this panel is displayed in the right upper corner - everything will still be grayed out.
- B) Part of the control panel indicating the presence of the various photos. Channels are represented by the color: white: no picture green: only Calcein photo present, blue: only Hoechst, cyan: both photos.

### Options available after loading

Now parts of the control panel are activated and you can click on two buttons ("Overview" and "Go") and select one of the radiobuttons to set the default to show in later activated windows (Blue & Green [BG], Blue [B], Green [G], Immigrant cells [Im] and Cells [Ce]). see the middle part of Fig. 1A.



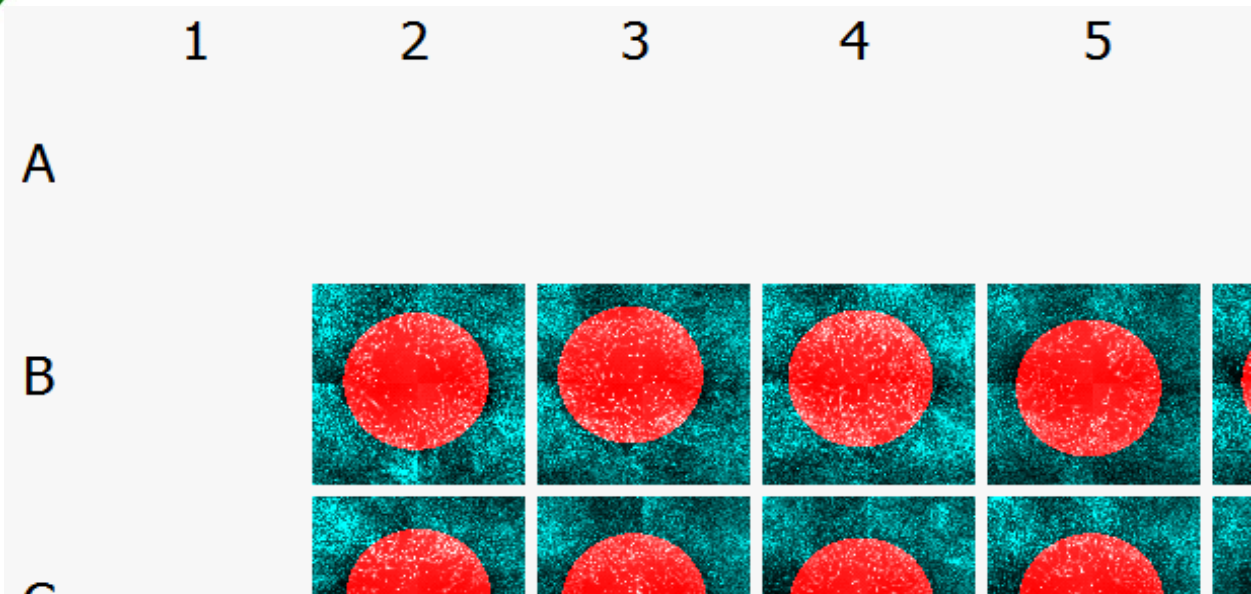


Fig. 2 The overview - left top part is shown, the photos of the first row and first column are either missing. or the user deselected them.

### The “Overview” button

If you click on the left most button you’d better be prepared to wait a few moments before you will see the results. In Fig. 2 the results are shown. It will show you all stitched photos in both colors and superimposed the predicted position of the original stopper in red. The overview is very handy to check the quality of the various wells and to decide whether all wells should be analyzed further.

### The “Go” button

This button will evaluate all selected wells (how to deselect/select wells see below) and output the results. To do this it will use various settings you set (again see below).



So **use this button in the end after you adapted all the settings** or when you have done all testing and the settings are all standardized and you go directly from loading the pictures to evaluating.

Clicking on the button will generate the following files:

In a newly created folder (called JPEGS) it will put a new composite photo showing **only** those cells that moved. The number of cells are written in the right lower corner. The file is in jpg format. One photo is created for every well.

A csv file containing all info about the photos and the cells therein. This file is perfect to load into a spreadsheet program. If you use Excel this file is still created but the generated data are automatically loaded into Excel (further details see below).

The radiobuttons to set the default to show

I think I will skip the description what the various selections will do to later. After you read the description of the various tabsheets most will become clear to you, so just read on.

Because now we'll come to the interesting part.

### Selecting / deselecting wells

Clicking on any of the colored wells in the control panel with the right mouse-button will deselect them; they turn red. Right-clicking them again will reactivate them and they will change color correspondingly. Any data from red wells will not be collected and shown when you click on either the "Overview" or "Go" buttons - see the corresponding entries on page 6.

### Showing the data for a single well and setting the parameters for analyzing.

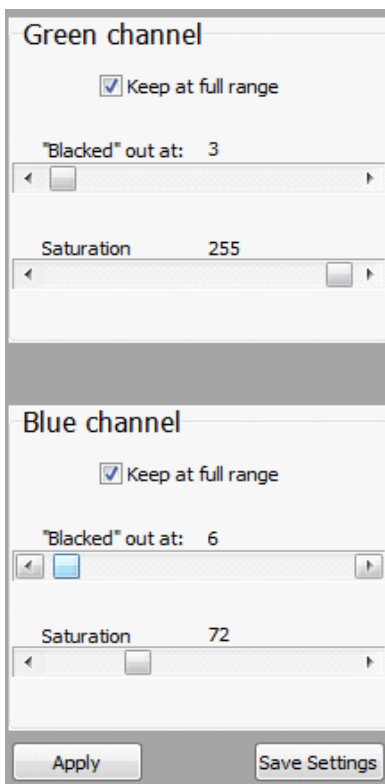
You can see all data for a single colored well by just clicking on it with the left mouse-button.

What you will see in the opening window depends on what you selected as default to show - see option on page 5.

The following is a description of the different tabsheets from left to right. Most people I know use the "Immigrant" tabsheet as default for setting parameters and viewing. As a matter of fact, after a period of trying out the different parts of the RingAssay program they use little else.

The tabsheet labelled:"Raw Two Channel"

Like the name suggests this tabsheet will show all 8 original photos united as a single picture in green and blue. The cell body will show up in green and the nucleus in blue.



As depicted in Fig. 3, on the right, next to the picture there is the possibility to enhance it. These kind of parameters can really improve the visual impression the photos make. They do little for the final evaluation of the picture by the RingAssay program. You can enhance the green and the blue channel independently. Both give you the possibility to correct for a light background by moving the upper slider to the right. A green sheen in the pictures can so be turned to black, likewise for a blue-ish background.

Weak signals can be enhanced by the bottom sliders, by moving them to the left the corresponding colors will be made brighter.

Both channels have the "Keep at full range" checkbox. When checked the grayscale of the original pictures is recalculated to show a smooth transmission of all remaining shades.

Fig. 3 The control panel of the "Raw Two Channel" tabsheet





This will result in smooth transmissions of the green and blue hues used in the final pictures. To see the differences just put the upper slider up, the bottom slider down and toggle the checkbox. Especially the green channel shows this effect.

Underneath the slider-boxes there are two buttons - you will find copies of these buttons on most of the other tabsheets as well.

#### The “Apply” button

This button will take the settings you just set (the four sliders and the checkboxes) and apply them to all other windows you might have opened by clicking on various wells. These new settings will then also be used if you click on the “Go” button - see page 6.

It is possible to evaluate every well with different settings, that is very easy; just open the wells and evaluate them individually. But most of the time you will want to have them all the same so you can really compare them. So the idea is play around with a few wells, test a few settings on the various tabsheets and if you find setting you like; click on the “Apply” button and then on the “Go” button.

If you click on “Go” without using the apply button first, the program will use the settings of the activated window for all wells that are not opened in a window at that time. The wells corresponding to other open windows will be evaluated by the settings of those windows.

This will enable you to use a different set of parameters on a small group of wells.

#### The “Save” button

This button enables you to save your settings for future use.

Not all your experiments will be using the same cells or the same batch of stoppers etc., etc. So it is handy to save your setting say like in a file named HeLa\_CheapStoppers3.RAS. Another file could be PrimaryCells\_DeLuxeStoppers.RAS. All these files will have the .RAS extension (RingAssaySettings).



If you want to use these settings later, you can use the menu item “Open Settings”.

#### The tabsheet labelled: “Blue Channel”

The second tabsheet shows exactly that. It stitches the four photos together and shows them as they are. I included this tabsheet just to get an overview to judge the spread of the cells. The green channel is most of the time so dominant that the nuclei are hard to see even with manipulation of the settings. There are no parameters to set so no “Save” or “Apply” buttons.

#### The tabsheet labelled: “Green Channel”

The third tabsheet shows the green photos -surprise, surprise. More importantly, the small control panel (see Fig. 4) gives you the possibility to set the radius of the stopper the program uses. This parameter is used to calculate the area of those cells that must have moved. Another thing you can play with is the exact position of the afore mentioned area.



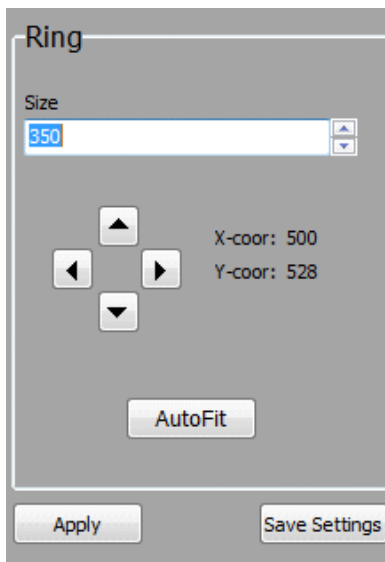


Fig. 4 The control options for the “Green Channel”

You can always go back to the calculated position by clicking on the “AutoFit” button. The calculated area is represented in the picture by the big red circle.

Again you will find two buttons labelled “Apply” and “Save” these have the same function as the two buttons with the same label on the “Raw Two channel” tabsheet - for an description see page 8.

### The tabsheet labelled: “Immigrant Cells”

Probably the most important tabsheet. Here can see directly the results and if you change the parameters the results will be updated instantly. Almost all parameters can be set on this page. However, some settings are even unique, see Fig. 5.

The control panels are copies to the corresponding panels described earlier. So the “Green channel” and the “Blue channel” are directly linked to those of “Raw Two channel” tabsheet. So for a description of the various functions see page 7. Again changing the settings will result in changing the picture but here you can also see their influence on the results.

The “Apply” and “Save Settings” buttons are linked to their counterparts on the other tabsheets, so they are described on page 8.

New is the control panel called “Nuclei”. Again there are two sliders controlling the green and red channel. This time however, they control just the area around the nuclei. It gives you an handle on another phenomenon; that of small objects that you can find in a lot of cell cultures. These might be left after some cells have died and lysed. Most of the time they are very predominant in the blue channel, even brighter than the real nuclei. They might be leaking into the green channel as well. With the two sliders you can correct for these objects.

To see which nuclei are passing all your tests and are counted is easy to see: these nucle turn **red** in the main

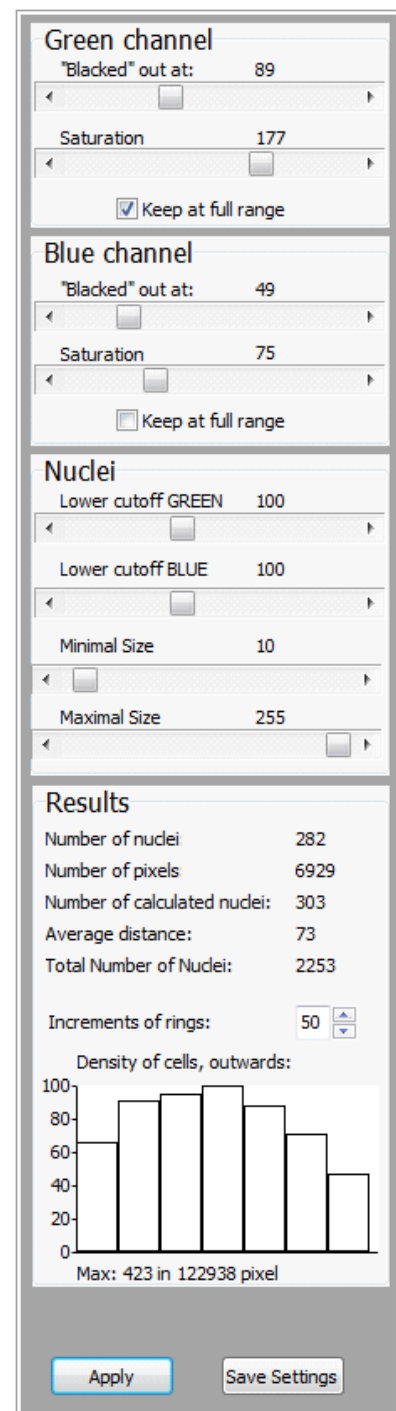


Fig. 5 The control and Results panels of the “Immigrant Cells” tabsheet.





picture. Those nuclei that fail stay blue.

A second way to correct for these is the setting of the minimal and maximum size a nucleus can have with the correspondingly labelled sliders. These can also be used to exclude bloated cells.

The panel called “Results”

This is the final panel where you can read the results for your chosen well. It will list the following data:

- **The number of nuclei** that have crawled within the border of the big red dot you defined on the “Green channel” tabsheet. Most of the time this is the only number you want to know for a certain well.
- **The number of pixels.** This is part of a build-in control it will be used to calculate
- **The number of calculated nuclei.** By using the number of blue pixels and the size parameters of the nuclei the program calculates how many nuclei should be there. If this number differs too much from the counted number of nuclei there is probably something in the well that gives a strong signal. Do have a look whether this is true and try to filter it out by changing the various parameters.
- **Average distance.** This is the average distance the nuclei are from the border of the big red spot. If the radius thereof was set correctly this is equal to the average distance the cells crawled. Always assuming they moved in a straight radial line - which they most certainly didn't. Still it is a good indicator for mobility: the higher the number the higher the average speed of the cells.
- **Total number of nuclei.** Just the total number of cells in all four original photos, so you can say something about density of the cells and crowding.

The bottom part of the “Results” panel is filled by a histogram. The width of the single bars is controlled by the number above the histogram. It is a figure showing the density distribution of the cells **outside** of the big red spot.

For understanding please look at Fig 6A. It depicts the four parts of the photo and the big red spot. Around this spot circles are drawn, see Fig 6B. Every next circle will have its radius increased. For clarity not all circles are drawn. The area between two circles is calculated. If a circle is partly outside the photographed area this is compensated for. The number of cells laying between the two circles is counted and the density (cells/area) is calculated and depicted in the histogram, shown as on page 7. All the numbers are relative to the max density measured and expressed as percentage. The exact numbers are shown beneath the histogram.

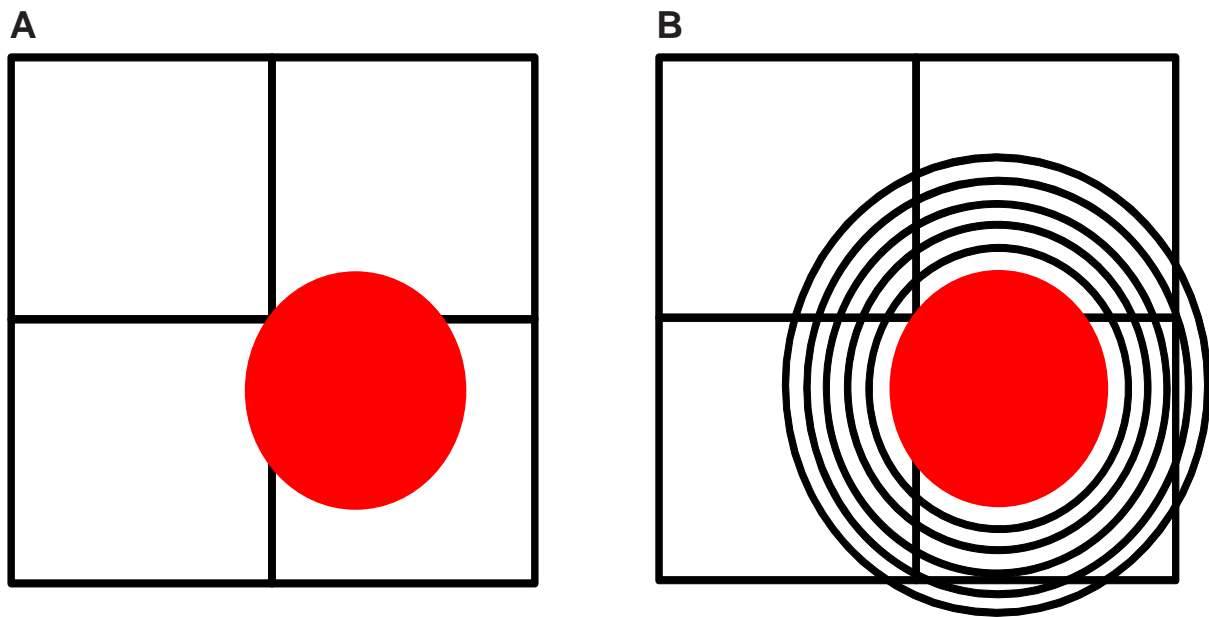


Fig. 6 The placing of the rings for the calculation of the histogram on the “Immigrant Cells” tabsheet.

This feature was included to help (but nothing more!) settle a discussion intrinsically linked to any mobility assay. It is the question whether what you see is mobility or growth. These two are hard to distinguish. Are cells moving into the free area or are the borders crowding daughter cells into the empty space?

The central idea was: if the borders are growing then the density of the area around the border should stay the same and only the new cells are moving in. You expect a flat histogram where all bars have approximately the same height.

If the cells are really moving and not dividing at all, you would expect the area around the red spot to be depleted of those cells that move in. The histogram should show that the first few circles show a lower density than those father away.

The density values of the outermost circles are very untrustworthy as the number of cells is high but the surface area is getting extremely small. This is caused by the fact that larger and larger parts of the circle do not overlap with the photographs at all.

So far the theory. Unfortunately in biology nothing is absolute. There are never going to be cells that only move and do not divide or only divide and do not move. At least the histogram will give you an indication of what might be predominant.

Most of you will now have the data they want, click on the “Go” button and look at the Excel sheet. Where all data about when and where, parameters, number of cells, average distances and cell densities are kept. However, there is another tabsheet. It gives some extra info about your cells that have nothing to do with mobility.

The tabsheet labelled: “Cells”

Like mentioned before this tabsheet does not show any data about mobility. It shows some data about the morphology of the cells. We use the ringassay to check which circumstances might influence the mobility of several different cell (lines). So we do a lot of drug-testing. One of the things we noted is that sometimes not only the mobility of the cells change but also their shape. So here it is my first attempt to look at this aspect of the





ringassay. So look at this tabsheet by all means.

You will be familiar with the look of the different tabsheets by now so most will look like expected. However, there are some differences. This tabsheet is not calculated automatically. Although the picture is shown all parts of the control panel are grayed out. There is also a part left of the picture with some colored headings. See Fig. 7A & B.

The reason for this behaviour is that the calculations for the morphology of the cells takes quite some time. So it is not done automatically but only if you want it.

To activate this part, click on the checkbox labeled: "Look at Cell level". A lot will happen, all at once. The control panel will be activated and look something like in Fig. 7B, The picture will have outlined cells like in Fig. 8A and beneath the colored buttons of Fig. 7A a lot of small pictures are listed see Fig. 8C.

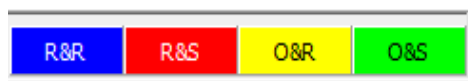
So, what do you see here? Let's start with the control panel. Again some sliders. I think "Green channel" and "Cells" - Min. and Max. size won't give you any problems. Neither will the indicator telling you how many cells are recognized with these settings out of the total. The program knows how many cells there are by assuming that the number of *bona fida* nuclei it counted before will equal the total number of cells.

Not all cells can be resolved as easily as nuclei. The cytoplasm tends to overlap, especially in photos that are slightly overexposed. You can try to compensate with the setting of the "Green channel". You can always see what cells can not be resolved by clicking on "Color". The picture will change as shown in Fig. 8A to 8B. Cells that can not be resolved will have the same color in the picture.

It is not hard to resolve the cells in the border area. The "watershed" method will do this very nicely. However, the data generated are not very good. Cells are round and smooth. This is logical; if there are many cells in a small area they tend to squeeze each other. So their shape is no longer defined solely by internal forces but also by external ones. My personal guess is that mathematically speaking they will end up with an hexagonal shape. So the RingAssay program uses only "free" cells to evaluate the shape and orientation.

Fig. 7A) Four colored buttons that are displayed left of the picture and B) the control panel of the "Cells" tabsheet. The control panel is shown in its activated state.

A



B

**Look at Cell level**

**Green channel**  
Cut off at: 134

**Cells**  
Min. size of cells 30  
Max. size of cells 2000  
158 c./276 tot

Border  Color

**Shape & Orientation**  
Ratio long/short: 3  
Coeff of Smooth: 0.75  
Elongation: 3.62 Ava.: 3.97  
Smoothness: 0.80 Ava.: 0.79

**Pie Chart Data:**

Color	Count
Blue	3
Red	47
Yellow	40
Green	68

**Legend:**

Color	Shape	Count
Blue	Round & Rough	3
Red	Round & Smooth	47
Yellow	Oval & Rough	40
Green	Oval & Smooth	68

**Distribution of radical orientation:** 66%

Save Welldata Apply

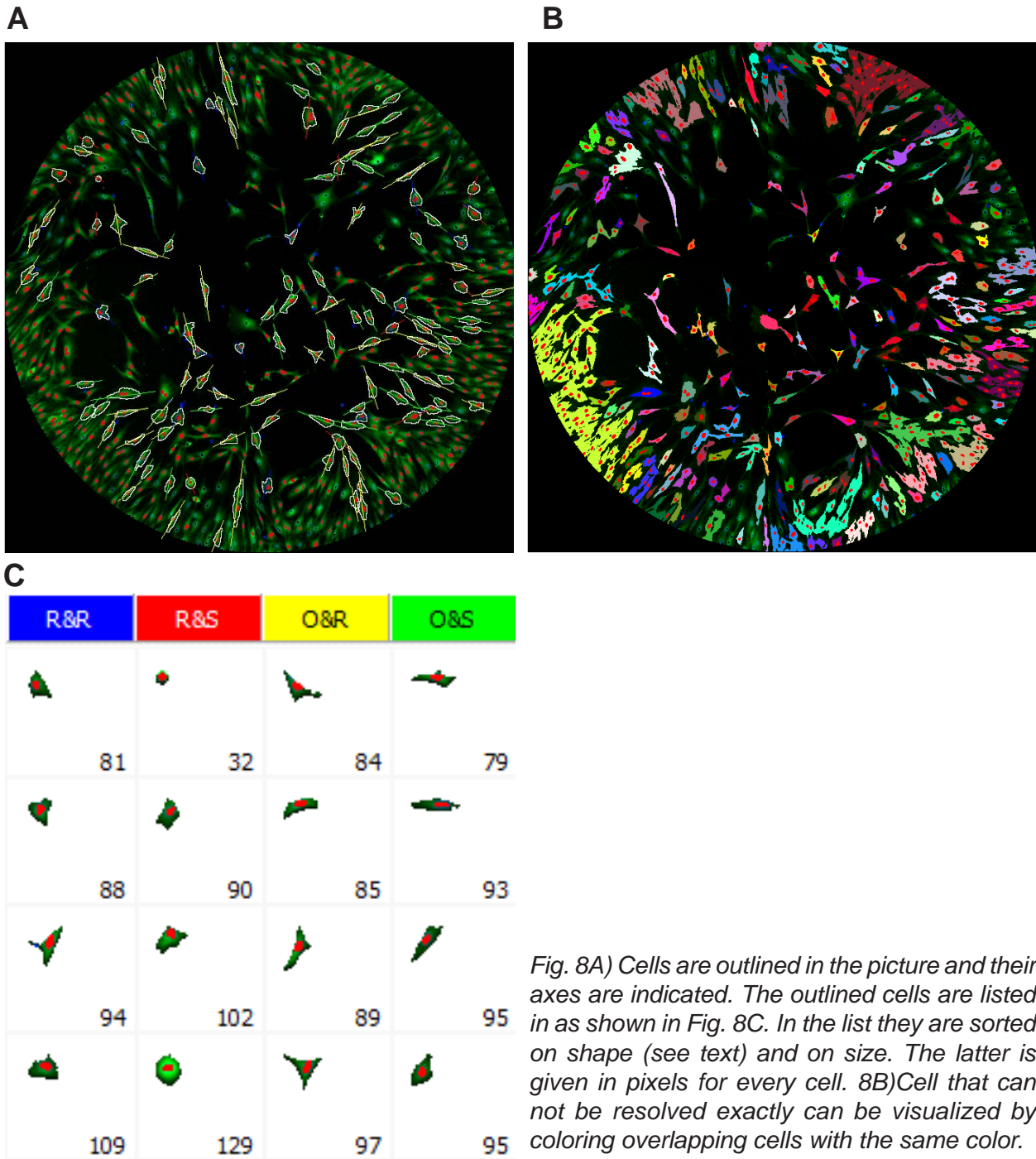


Fig. 8A) Cells are outlined in the picture and their axes are indicated. The outlined cells are listed in as shown in Fig. 8C. In the list they are sorted on shape (see text) and on size. The latter is given in pixels for every cell. 8B) Cell that can not be resolved exactly can be visualized by coloring overlapping cells with the same color.

The panel called “Shape and Orientation”

But let us get back to the “Border” setting, giving us the outlined cells. The next part contains the settings and results for “Shape and Orientation”. At the moment there are only two parameters for the shape of a cell. First, are they elongated (oval) or rather round? Secondly, how is their border, smooth or rough?

Both characteristics are a matter of definition. In Fig. 7B Oval is defined as having a length of greater or equal to three times the width. You can change this by just moving the slider. Everything will be updated directly.

The smoothness of a cell can also be defined. In this case there is a single coefficient that defines smoothness and roughness. Mathematically the polar coordinates of the different pixels that make up the border are plotted onto a polynome. The correlation between the





real points and the polynome is used for the definition of this parameter.

Right below the sliders both the median and average of the elongation and the smoothness are given.

The data is depicted in several ways. The listing of the individual cells on the left of the window is the most obvious way. The cells are sorted in four categories according to their shape and smoothness. Round & Rough (Blue), Round & Smooth (Red), Oval & Rough (Yellow) and Oval & Smooth (Green). Furthermore, the cells are sorted to size, the small ones on the top and the bigger on the bottom. Using the same color scheme the numbers of cells that belong to one of the categories are shown in the pie-chart. The numbers are written on the right of the pie-chart. Color codes are beneath.

The lower part of the control panel is showing data on the orientation of the cells. As the cells crawl inward from the border of the well towards the midpoint I thought it might be interesting whether their main axis (cells only have this when they are not round!) has any orientation at all. The main axes of every cell is depicted - see Fig. 8A. The angle between this axis and a direct line towards the centre of the big red spot is determined for all cells. These can be numbers between 0 (cell is directly pointing at the centre) and 90 degrees. The values are used for the generation of the histogram at the bottom of Fig. 7B. Again all the values are given in percent. The percentage of cells that show an angle less than 45 degrees is shown over the histogram.

The final controls are at the very bottom. Clicking the left button labelled "Save Welldata" will create a .csv file that can be loaded into any spreadsheet program and it will also save the main picture in jpg format.

The right button, "Apply" is again responsible for setting the parameters you choose on all other windows.

