Nemo user guide

A tool to analyze genes and chromosome territories
distributions from 3D-FISH experiments

Version 1.1

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Nemo user guide

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1   Background

Cell imaging techniques are integral to successful life science studies and, at the dawn of the post-genome sequencing era, techniques that permit the accurate localization of genes and their products within cells and tissues will be in ever-increasing demand. The rapidly developing field of cell imaging techniques has greatly contributed to the current understanding of cell structure and function. Three dimensional fluorescence in situ hybridization, 3D-FISH, is used to study the organization and positioning of chromosomes, sub chromosomal regions, and/or specific sequences such as genes or RNA in cellular preparations by hybridization of probes with complementary sequences in cells chromatin preserved 3D structure.

2   Introduction

Many different programs allow 3D-FISH experiment image analysis (commercials or free), one of the more efficient programs to process 3D-FISH confocal microscopy images is Smart 3D-FISH, an ImageJ plug-in designed to automatically analyze distances between gene spots. This ImageJ plug-in provides a powerful automatic processing mode able to detect and measure object distances in multiple-channel experiments. After processing, Smart 3D-FISH uses predefined parameters (for objects and images) to auto estimate segmentation quality. Results are then manually checked using various produced text and image files. That point was a problem with Smart 3D-FISH because of its poor user interface, the spread of data in various text and image files also made this post processing step time-consuming. We decided to develop new software, NEMO, a Smart 3D-FISH graphical user interface providing all data in one place to improve result investigation, giving user ability to change some of the automatic processing parameters and adding some basic data management functionalities. We also tuned Smart 3D-FISH to better detect chromosome territories

3   Architecture

Nemo is a Java application using many java libraries but specially the Smart 3D-FISH library developed by Thomas Boudier which uses the *ImageJ* library (Wayne Rasband). To avoid any Java version problem, a Java runtime environment (1.6) is bundled into *Nemo* installation package (*jre* directory).

4 Installation

Nemo can be used under Windows and Linux 32 bits platforms (Macintosh and 64 bits packages will come later), each version can be freely downloaded from https://www-lgc.toulouse.inra.fr web site *Nemo* section.

4.1 Windows 32 bits installation

Download the *Nemo-Win32.zip* file from the web site, unzip this zip archive whatever you want on your hard drive but in a place where you have write access (a log file will be generated at this place). That's it! You can run Nemo by double-click on *nemo.exe* file.

## 4.2 Linux 32 bits installation

Download the *Nemo-Linux32.tar.gz* file from the web site, unzip this zip archive whatever you want on your hard drive but in a place where you have write access (a log file will be generated at this place). That's it! You can run Nemo by executing the *RunNemo.sh* file (if this file is not executable, run *chmod a+x ./RunNemo.sh* or use you desktop manager interface.

5 Preferences setup

Open the preferences dialog (File->Preferences)
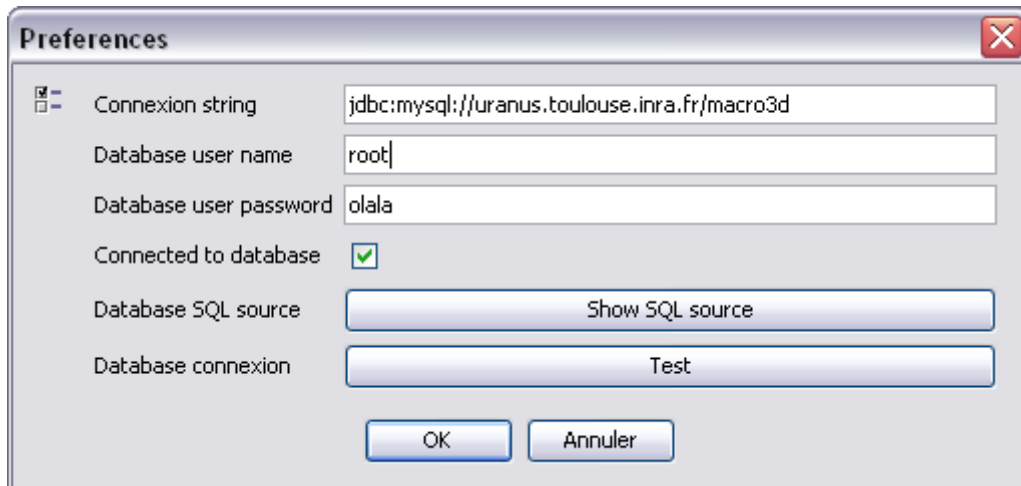
You can get the SQL source code by clicking on the *Show SQL source,* a popup window provide complete SQL source you can copy and paste into your MySQL management tool to create database tables.

After database tables are created, you can provide the connection string, user name and password to let Nemo connect to database. Click on the *test* button to check Nemo can reach database.

NB: considering following connection string : *jdbc:mysql://uranus.toulouse.inra.fr/macro3d*
    uranus.toulouse.inra.fr is the MySQL server name
    macro3d is the database name

The *Connected to database* checkbox disable connection when unchecked.

Creating and opening fields
If you want your data be stored into a MySQL database, you must first create the database on your local computer (using XAMPP or any other local MySQL package) or you can use any available MySQL LAN server (ask you system administrator). The SQL source code to create database tables can be copy from the Nemo preferences dialog

6 Creating and opening fields

6.1 Creating a new field

A field is a set of cell lying on the same support and acquired in the same images. Before processing individual cells, you must create a Nemo field to import images and setup processing parameters. Open the New Field dialog (File->New Field …)

A field is a file system directory containing images and data in dedicated subfolder using Nemo predefined names. Three steps are to be complete to create a field:

6.1.1 Field name and path
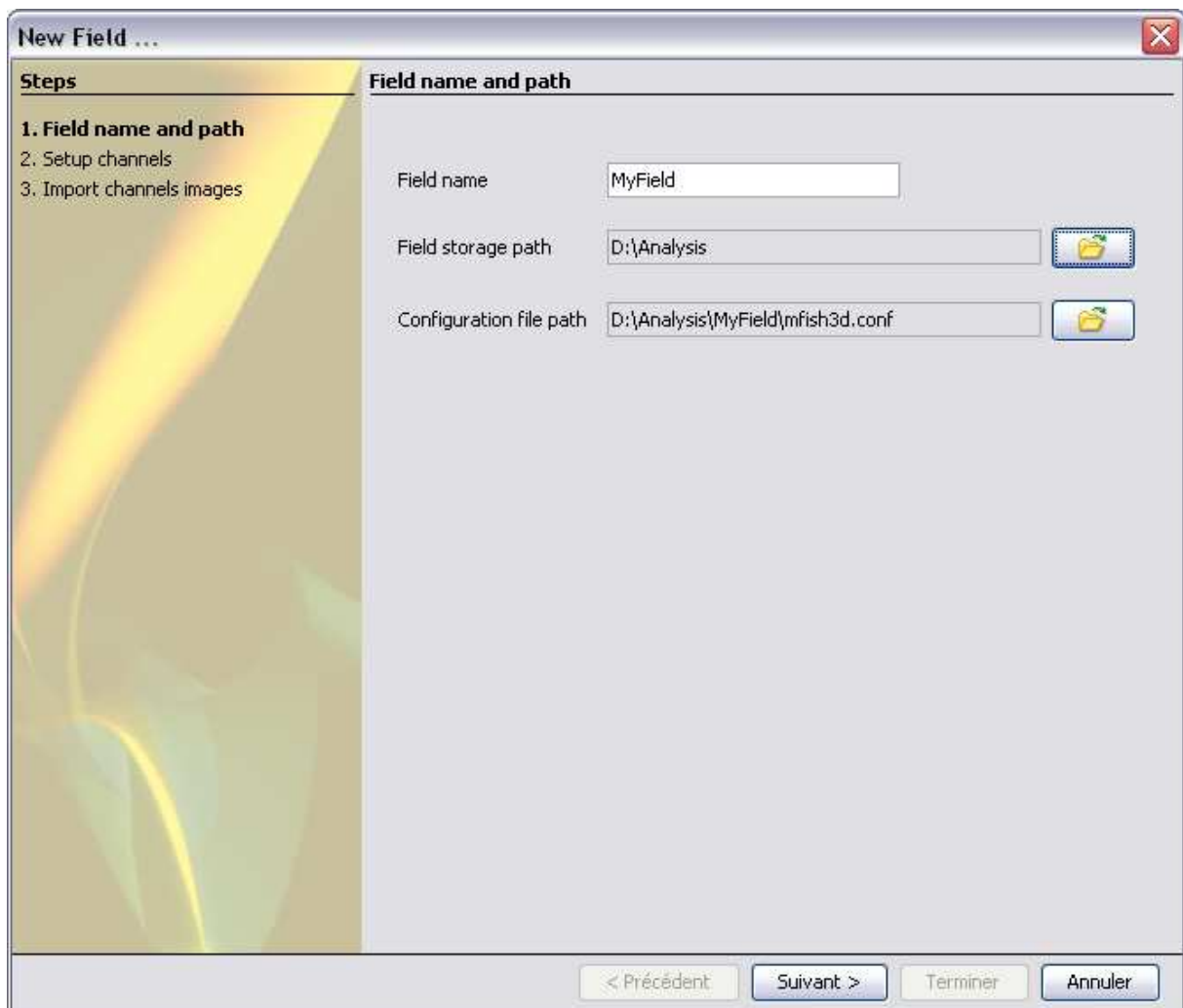
Field name text is used to create a directory in the *Field storage path* folder. The field configuration file is automatically named *mfish3d.conf* and is stored in the field directory. As an example, the values used in the screen dump will create a directory named *MyField* in the *D:\Analysis* folder, the field configuration file, *mfish3d.conf*, will be then stored in this field directory (*D:\Analysis\MyField*). **For Nemo, a field is a directory**.

NB:
The configuration file is stored by default in the field directory but it can be named differently and stored in another place. By example, user can decide to store all configuration files in a dedicated directory.

6.1.2 Setup channels
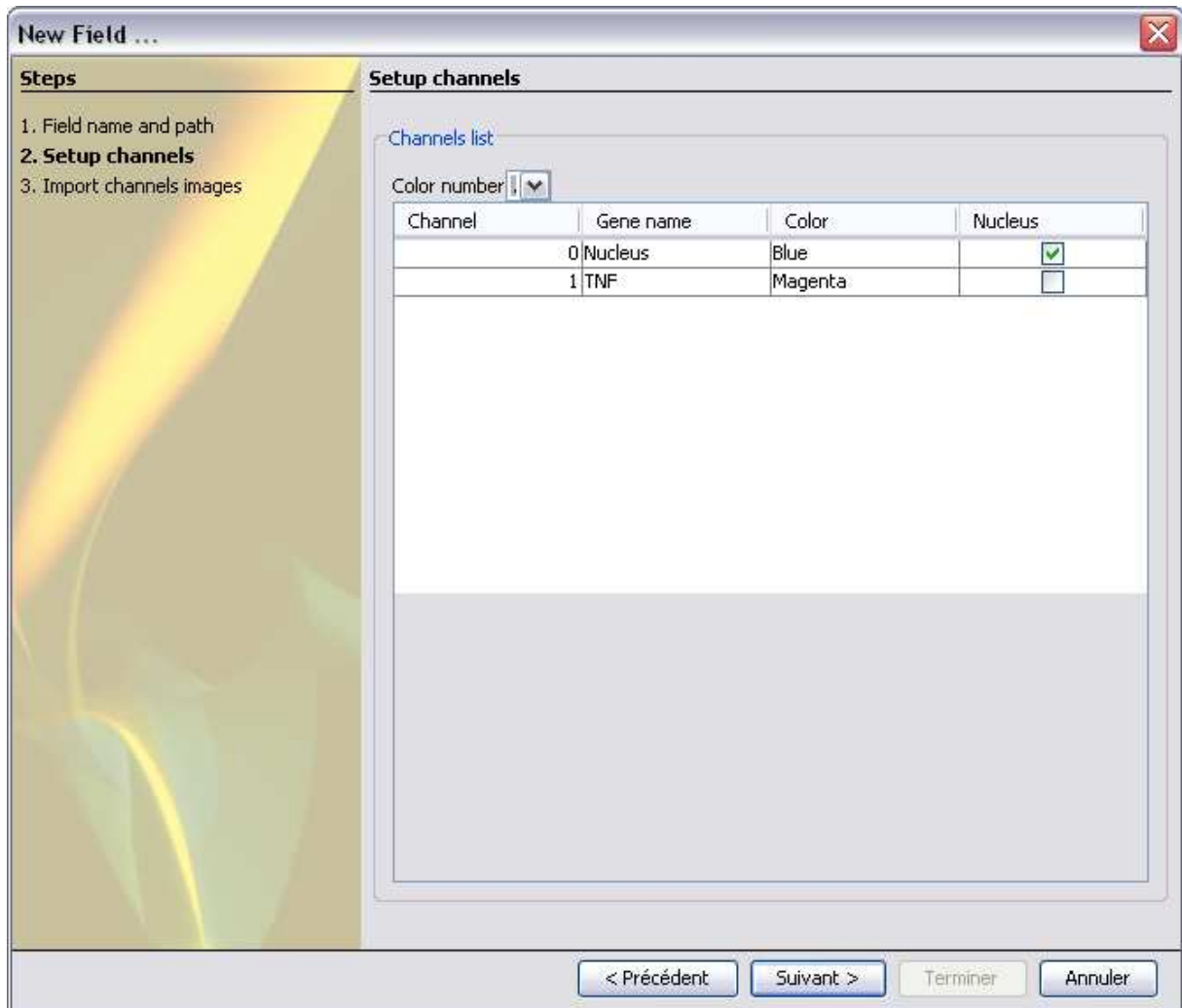
This page is used to provide channel number, colour and names. One "Nucleus" channel is mandatory because Nemo need a "container channel" to process some measurements (angle, local ray). The nucleus channel is processed differently since it only one object is assumed to be bigger than others.

NB:
Nemo better process chromosome territories if channel name is prefixed by "TC_" string, this tag will activate some changes in image processing:
- TopHat 3D filter is disabled
- One automatic segmentation threshold value calculation parameter is different than for genes.

6.1.3 Import channels images
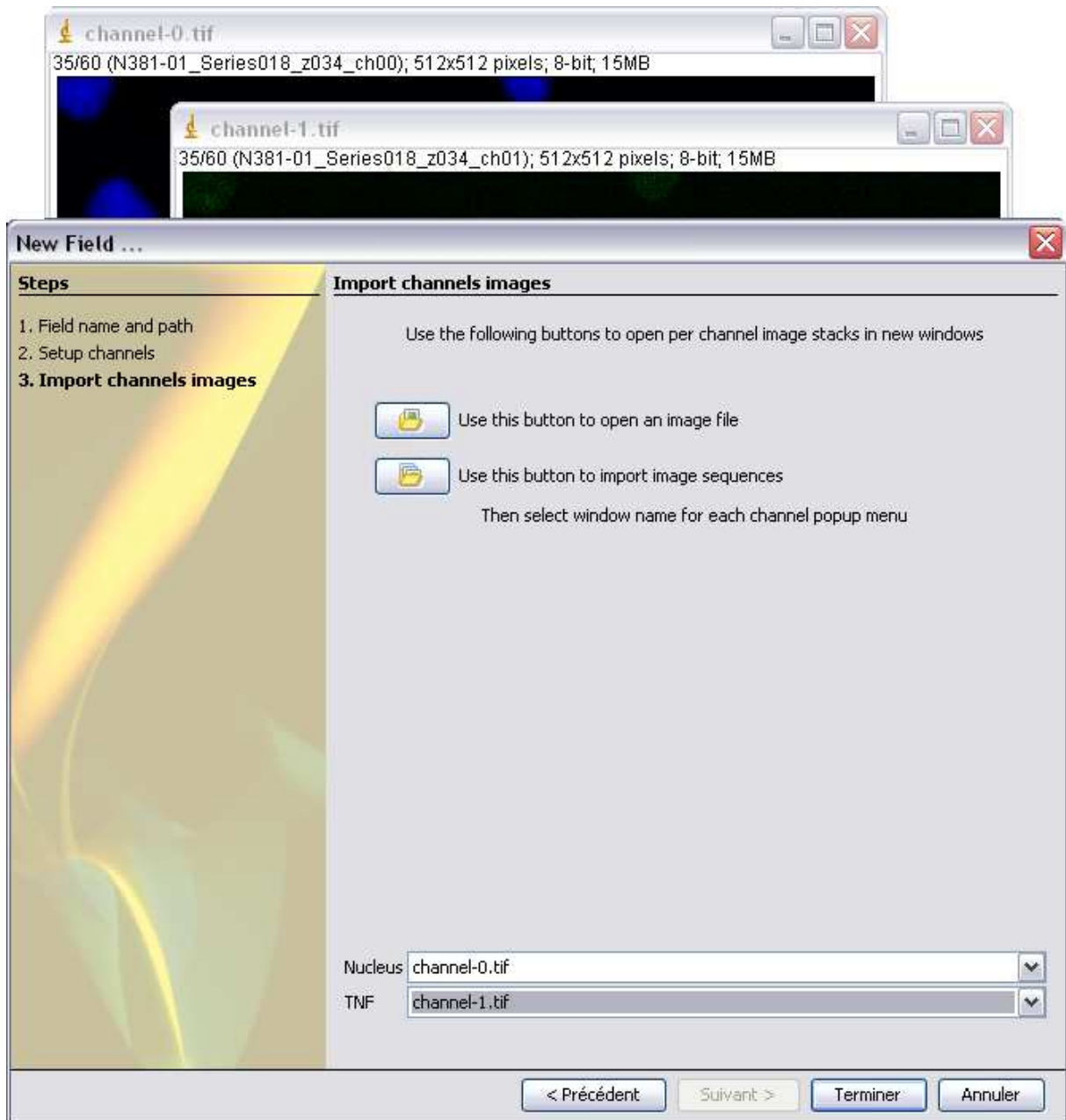
This step provides per channel images stack import. .8-bit or 16-bit grey level TIFF image stacks can be imported for each channel (additional image formats will be supported in a future release). NEMO can process epifluorescence (deconvolved or not) and confocal stacks but results pertinence will always depends on raw image quality

User open one stack window per channel and then select corresponding windows name for each channel using the bottom popup menus. Click on "Terminate" button to create the new field.

NB:
Do not close stack windows before the new field is displayed in Nemo main panel.

6.2 Opening an existing field

You can open any Nemo existing field by opening it configuration file: use the File->open menu, navigate to the configuration file and click on open button. After a loading waiting time (depending on the number of field cell), you will get the Nemo main panel showing your field. You can also use the *File->recents* menu to quickly re-open recently opened fields.

7 Adding individual cells from fields

You can define each cell by manually drawing a region of interest (ROI) onto the field image. This ROI is used to produce a sub image representing only that cell for further processing. NEMO also provides a function that detects automatically cells in fields taking into account circularity and minimum area parameters for individual nuclei. Each added cell is represented in the small cell outline image (above the *add cell* button).You can also display this image in a dedicated resizable window by a single click in the small cell outline image.

7.1 Manual cut

Open the field stack by using the *Cell->Add cell from field* menu; use one of the tools from toolbar to draw one ROI representing one cell.
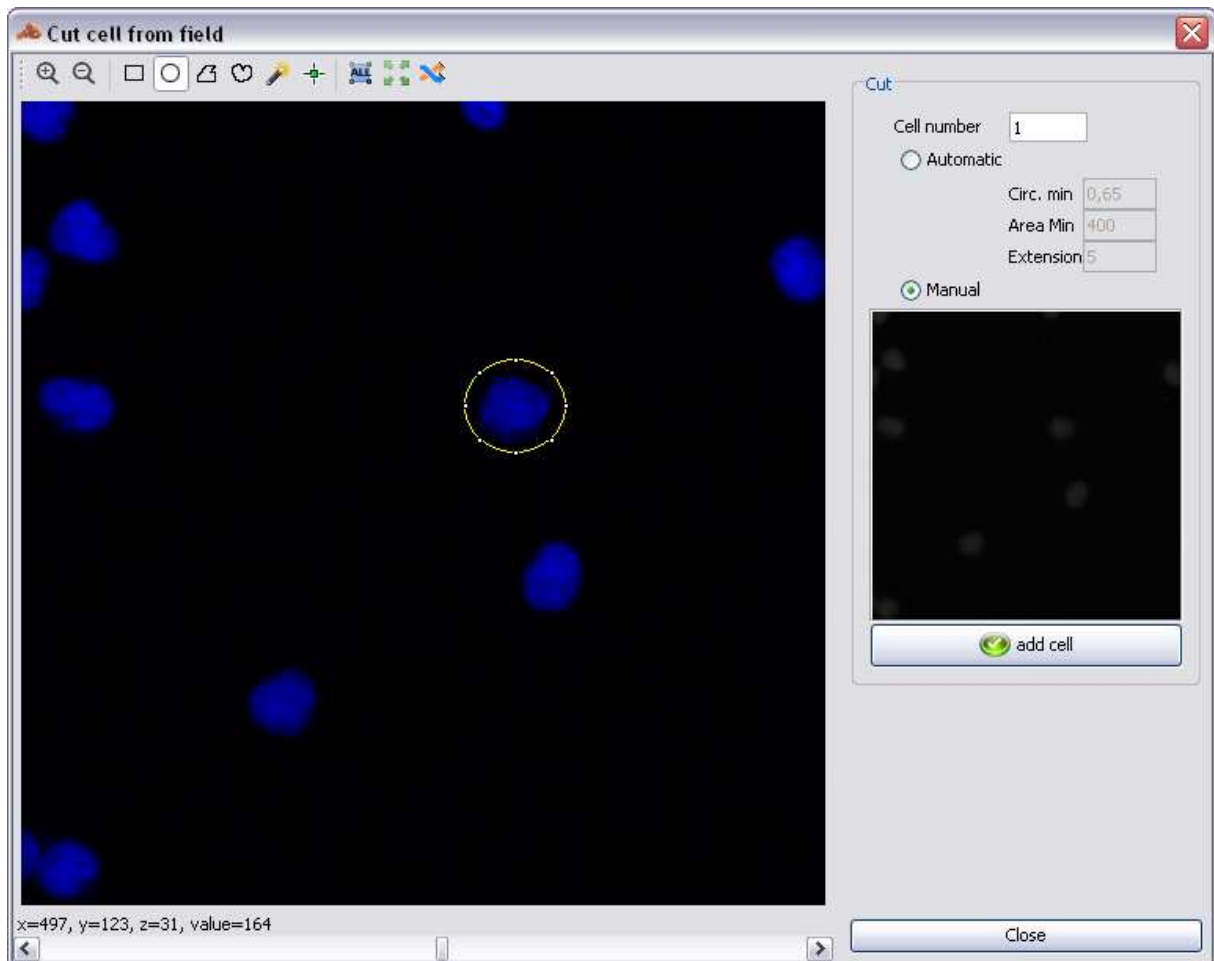
🔍	Zoom in	▭	Rectangular selection	⬠	Polygonal selection
🔍	Zoom out	◯	Circular selection	♡	Free selection
🪄	Magic wand	✛	Point selection	▦	Select all
⠿	Dilate/erode	✕	Invert selection		

You can move ROI using the keyboard arrow keys, ALT + arrows key enlarge/reduce ROI having the upper left fixed, press the CTRL + UP /CTRL + DOWN keys to dilate/erode ROI of one pixel.

After cell is outlined, click on the *add cell* button to let Nemo cutting a sub image representing only that cell for further processing. Each cell gets a numbered name (you can change in the *cell number* textbox if necessary); this number is automatically incremented by each cell addition.

7.2 Automatic cut

You can ask Nemo to automatically detect and cut cells for you: check the *automatic* radio button and click on the add *cell button*. Each cell ROI is detected and tested against 2 parameters:

- *Circularity min*: minimal circularity value to accept ROI. A circularity value of 1.0 indicates a perfect circle. As the value approaches 0, it indicates an increasingly elongated shape.
- *Area min*: minimum 2D area (in pixels) value to accept ROI

Each accepted ROI is then dilated using the *Extension* number of pixels to get some background for each cell (this dilation value is very image quality dependant).

8   Browsing cells

You can browse cell channel images by double-clicking cell name from the left panel tree view: each cell channel images are individualized in one stack, ready to be processed.
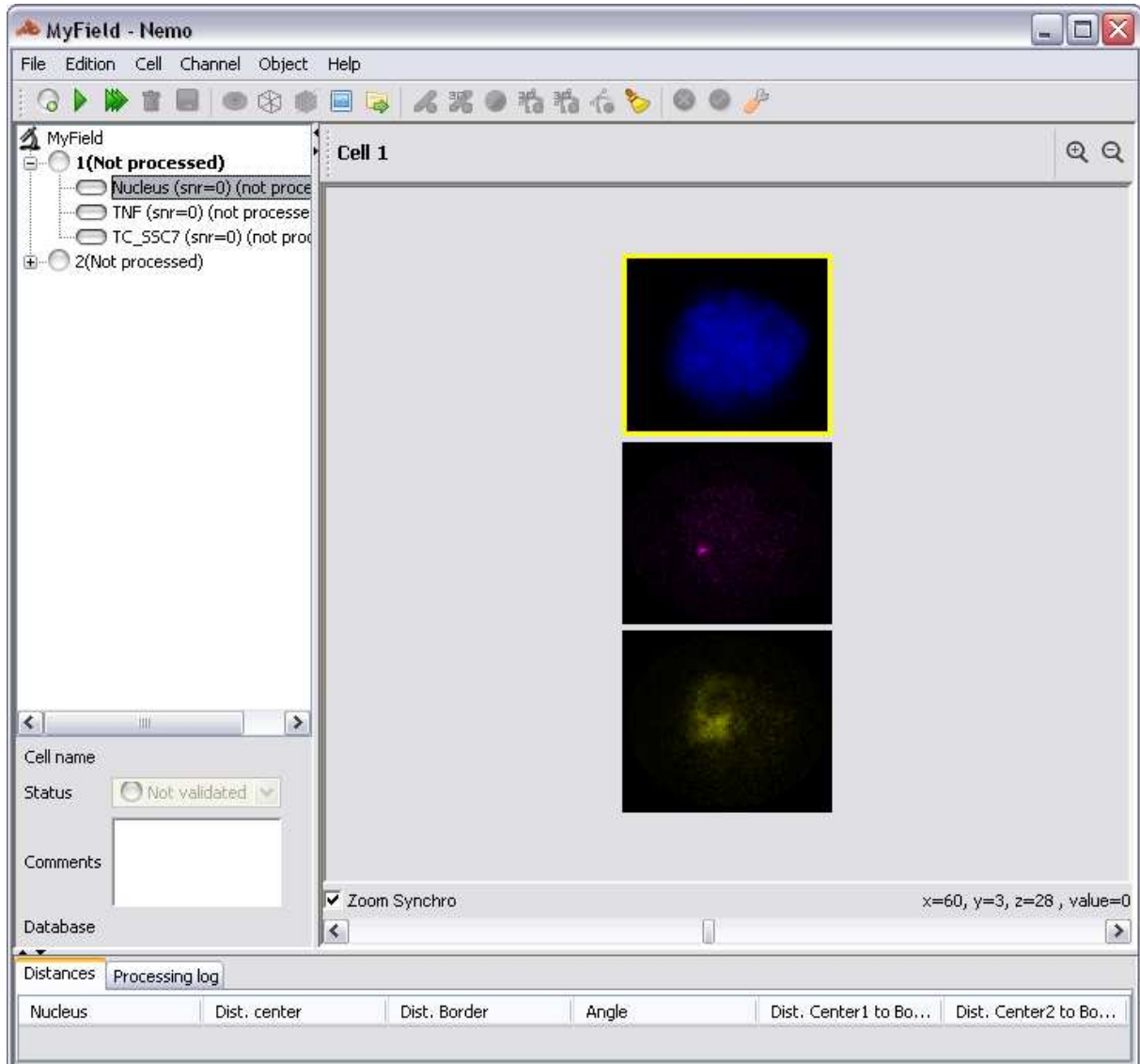
Use the horizontal scroll bar to explore stack Z dimension, you can zoom in and zoom out by using the upper right small icons. When the cell will be processed, a second image column will display per channel segmented images but before processing cells, you must setup field parameters.

NB:
Most of the field, cell, channel and object actions can be triggered by using toolbar buttons, main windows popup menus or tree view contextual menu.

9 How Nemo segments objects and provide distances

9.1 Segmentation method

In order to detect objects, NEMO first determine a global threshold on the image, this threshold differs according to the type of detected objects: spots, areas (like CTs) or whole nucleus. Maximum local pixels above this global threshold are computed and serve as seeds for objects. From each seed a local threshold is computed, all neighbourhood pixels above this local threshold are aggregated to the seed to form the object. Only the global threshold need to be adapted from one feature to another, the global threshold is based on a quantile of histogram. However this quantile could be a parameter (in a future Nemo release) in order to deal with the variety of images and features. Additionally a 3D median filter (radius = 2) is applied in order to reduce noise and improve segmentation. A 3D tophat filer (radius = 5) is applied to enhance spot detection by subtracting local background.

Nemo can use following 2 special settings that improve CTs segmentation:
      * The quantile value for CTs global threshold computation is set to 99.79 % instead of 99.98 % (default gene channel value). This quantile value has been manually adjusted using representative 3D chromosome segmentation experiment sets.
      * The TopHat3D filter is disabled for CTs

User must tag channel as "CT" channel to activate these special settings (see 9.3)

9.2 Distances

Distances are computed for each pair of objects. Distances are euclidean distances weighted by the xy and z resolution defined by the user. NEMO computes centre to centre distances between objects; it also computes the border of each object allowing the computation of centre to border distances and border to border distances. Border distances may be useful to compute for instance the distance of a spot to the membrane of the nucleus.

9.3 How to tag a channel as Chromosome Territory

Nemo special settings for CTs detection can be activated by using "TC_" prefix in channel name (e.g.: TC_SSC7). You can check Nemo will use CTs settings by verifying TopHat 3D filter is disabled and cannot be check (checkbox is disabled) in channel processing parameters. Log file also denote CTs settings usage.

10 Field parameters

Two parameter types are to be setup: segmentation estimation parameters (channel and object level) and processing parameters. Segmentation estimation parameters are used after processing to highlight unreliable results by specifying minimal image signal-to-noise ratio value, expected object number, minimum and maximum object volumes. Processing parameters define filters to run on raw images (Median 3D, TopHat 3D, mathematical morphologic 3D) and xyz resolutions Click on the 🔧 button from the toolbar or from the tree view field level contextual menu.
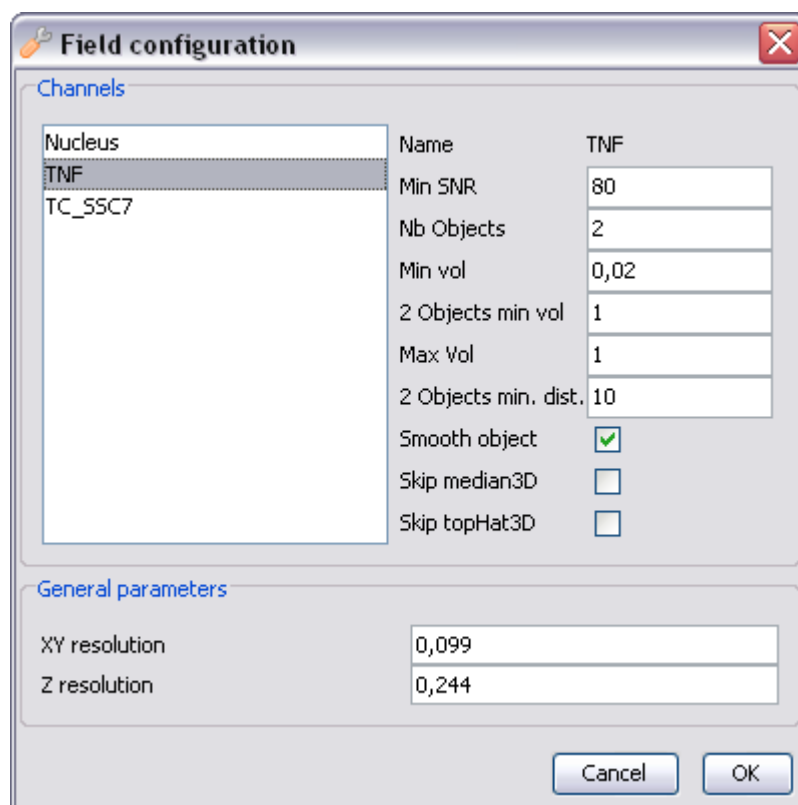
User select a channel in the left panel list to display or update selected channel parameters in the right panel:

10.1 Segmentation estimation parameters

10.1.1 Channel level:

Min SNR (Minimal Signal to Noise Ratio value)
Channel will be tagged with a *Low SNR* message if channel SNR value if lower *Min SNR.*. A Low SNR channel usually denotes a low quality image source.

*Nb objects (*Expected object number)

Channel will be tagged with *more objects* or *less object* by comparing detected object number with this value.

10.1.2   Object level

Min vol (Object minimum volume*)*
Object will be tagged with *small object* if it volume is lower than *Min vol*. This parameter is used to tag small object resulting from image small artifacts. Such objects are displayed but they are excluded from results (files and database) unless user explicitly accepts them.

Max Vol (Object maximum volume)
Object will be tagged with *big object* if it volume is greater than *Max vol*. This parameter is used to tag big object resulting from image big artifacts. Such objects are displayed but they are excluded from results (files and database) unless user explicitly accepts them.

10.2 Processing parameters

Smooth Object
Use a morpho-mathematical 3D filter to smooth objects (3D closing procedure)

Skip median 3D
Ask Nemo to skip Median3D filter

Skip TopHat 3D
Ask Nemo to skip TopHat 3D filter

XY resolution
X and Y dimension resolution value

Z resolution
Z dimension resolution value

TwoObjects min vol and *TwoObject min dist* parameters are used to select candidate objects for splitting into two smaller objects (gene chromatid, overlapping chromosome).
* *TwoObjects min vol (*Double object minimum volume)
  If object volume is greater or equal to *TwoObjects min vol* and lower or equal to *Max Vol*, Nemo will try to split it as 2 smaller objects. These two sub objects are then accepted only if their local maxima distance is greater than *TwoObjects min dist* (see below).

* *TwoObjects min dist (*Sub objects minimum distance)
  Minimal local maxima distance to accept sub objects coming from object split.

These volume and distance parameters cannot be correctly setup at the very first Nemo run (unless user knows exactly expecting objects size). User firstly applies default values and tune them regarding produced results.
**NB:** You can disable double object detection by setting the same value for *TwoObjects min vol* and *Max Vol.*

11 Processing cells

You can process a cell by using it tree view contextual menu or tool bar button ▶ .Processing a cell means that each channel is sequentially processed. If you want to tune some parameters for a single channel only, you can process only a single channel by using the same buttons having selecting the channel name in the tree view. The Process dialog title will reflect your tree view selection.
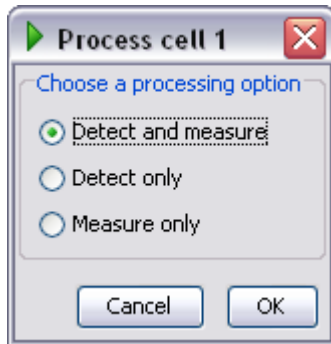
Cell 1 is selected   *Cell 1 TNF channel is selected*
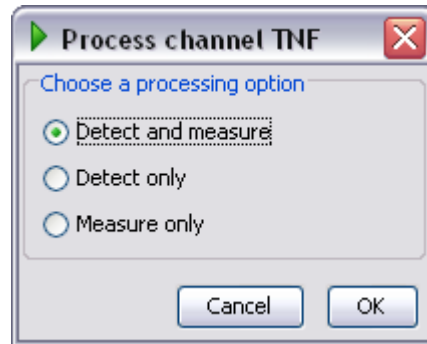
To process a channel, Nemo first detect objects and then measure various distances. Sometimes, channels objects cannot be automatically detected because of artefacts, bad image dynamic, etc. In that case, user can help Nemo using some tools (we will see later) which implies successive channel processing until the result is acceptable. In that situation, user can choose to only detect object without measuring to avoid measurement processing time. When the segmentation result is correct, user can choose to only measure distances (this can be done at the cell or channel level).

NB:

If some measure are not up to date with detected objects (user has run a new detection for a channel but has forget to re-run the measurement on the channel newly detected objects), stars characters (*) shows not up to date channels. Run a measure only processing to synchronize measurements with objects.
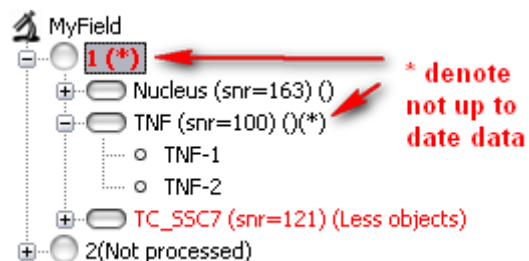
You can follow Nemo processing in the *Processing log* bottom tab, Nemo write a lot of things in this tab, this can help you to understand what is happening or why nothing happens (by example when Nemo performs longs task like TopHat and median filters, border to border and feret distances for big objects). Click on the cell in the tree view to see the cell log, click on the field to see the field log. These logs can act as tractability data and can be found in *.log* files in field folder.

12 Understanding results

Results are composed of object characteristics and various distances concerning pairs of objects. Results are stored in files at the time of validation but user can browse then using the bottom *Distance* table main panel.

12.1 Object characteristics

Object characteristics are shown when user click on object in tree view, here is the list:
Centroid XYZ coordinates, volume, area, compacity, local ray, Feret Distance. The local ray value is the length of the segment starting from the nucleus center, passing through the object center and ending at the nucleus border. Compacity, $(Volume^2 * 36 * PI) / Area^3$, is equal to 1 for perfect sphere and decrease to 0 for less spherical objects. Feret distance is the longest distance between any two points along the object. Volume, area and distances are calculated using the field configuration XY and Z resolutions

12.2 Distances

Various distance are displayed when user click on channel (homologous distances) or cell (heterologous distances). Homologous distances are distance between objects pairs from the same channel, homologous distance can only exists for channel having more than one object. Click on any channel to display homologous distances. Heterologous distances are distance between cell objects pairs from different channels. Click on cell to display heterologous distances. Each object pair distance is made of the following values: center to center, border to border, centre to border (2 combinations), angle (homologous distances only) and co-localization percentage (heterologous distances only). The border to border distance is the shortest distance between any two points along the objects borders (equal 0 when objects are connected). The angle value uses the nucleus center as origin. The co-localisation percentage is the smallest object into biggest object co-localisation ratio. You can select table lines using mouse and Ctrl-C to copy data to clipboard.

13 Helping Nemo to better detect objects

Nemo detect objects in two steps: image maximum pixels are detected using a histogram based threshold value; these pixels are then used as seed for 3D connectivity algorithm combined with local threshold. The histogram based threshold value is automatically calculated by Nemo using fixed quantile values: 99.98% for genes and 99.79% for chromosomes territories, these values were computed by estimating corresponding objects volumes and numbers in cells (up to 4 spots for genes and 2 spots for chromosome territories). There are many ways to help Nemo to better detect objects: user can change segmentation threshold, mask some image parts, provide a manually positioned seed and finally user can draw object using per slice ROI into images.

13.1 Changing segmentation threshold

In some images, Nemo can miss or over estimate some objects, this problem usually come from a shifted threshold value caused by noisy or low dynamic images. In that case, user can manually change the threshold using the set threshold dialog (Channel menu->Set channel Threshold) for the channel.
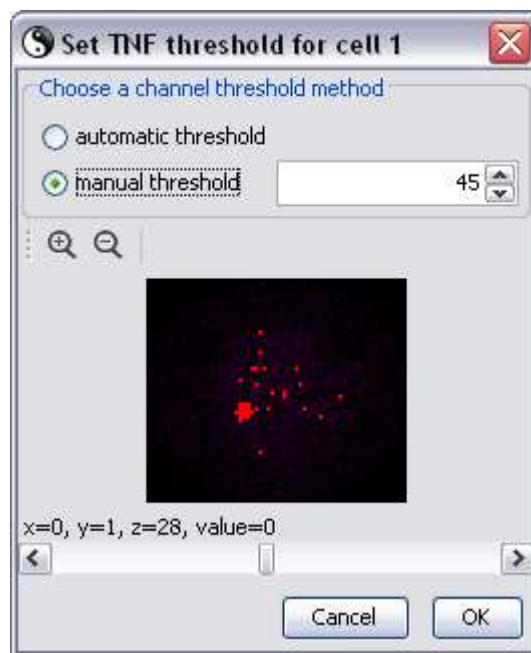
This dialog displays the threshold image to help user setting up the manual value. Reducing the threshold value can make missing object appears, increasing threshold value will reduce threshold pixel number, this can potentially change the pixel seed localisation so final object can be quite different.

13.2 Masking image parts

Images can be degraded by signals user knows they are artifacts (probes in cytoplasm, RNA residues, etc.). This kind of image generally cannot be automatically processed because of the histogram perturbation; one method to potentially fix the problem is to mask these artifacts. Nemo provide a non destructive tool to mask image parts, per slice or for all slices. This mask can be applied to one channel only or all channels.

13.2.1 Edit mask

Select the channel you want to mask images and use the *Edit image mask* contextual menu: The following dialog appears:
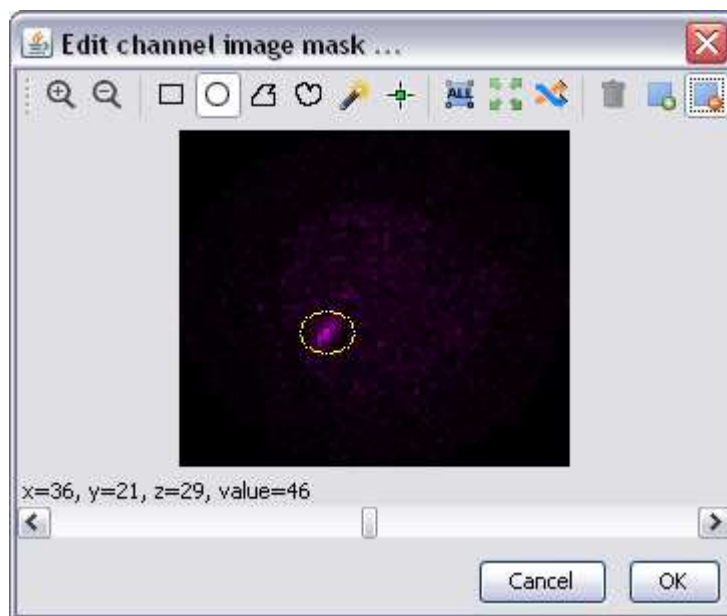

Select the zone you want to mask using any selection tool and click on the ▣ button to exclude this zone. If it is easier to define zone to keep than zone to exclude, select the zone to keep and click on the ▣ button. All defined zone are included or excluded, they cannot be mixed. After clicking on the OK button, Nemo display the defined mask in main view, if you choose to exclude zones, Nemo will use red border to denote zone to mask. If you choose to draw zones to keep only for processing, Nemo will use green borders. In any cases, a small blue circle will be displayed on the masked channel tree view icon (or cell icon if entire cell was masked).
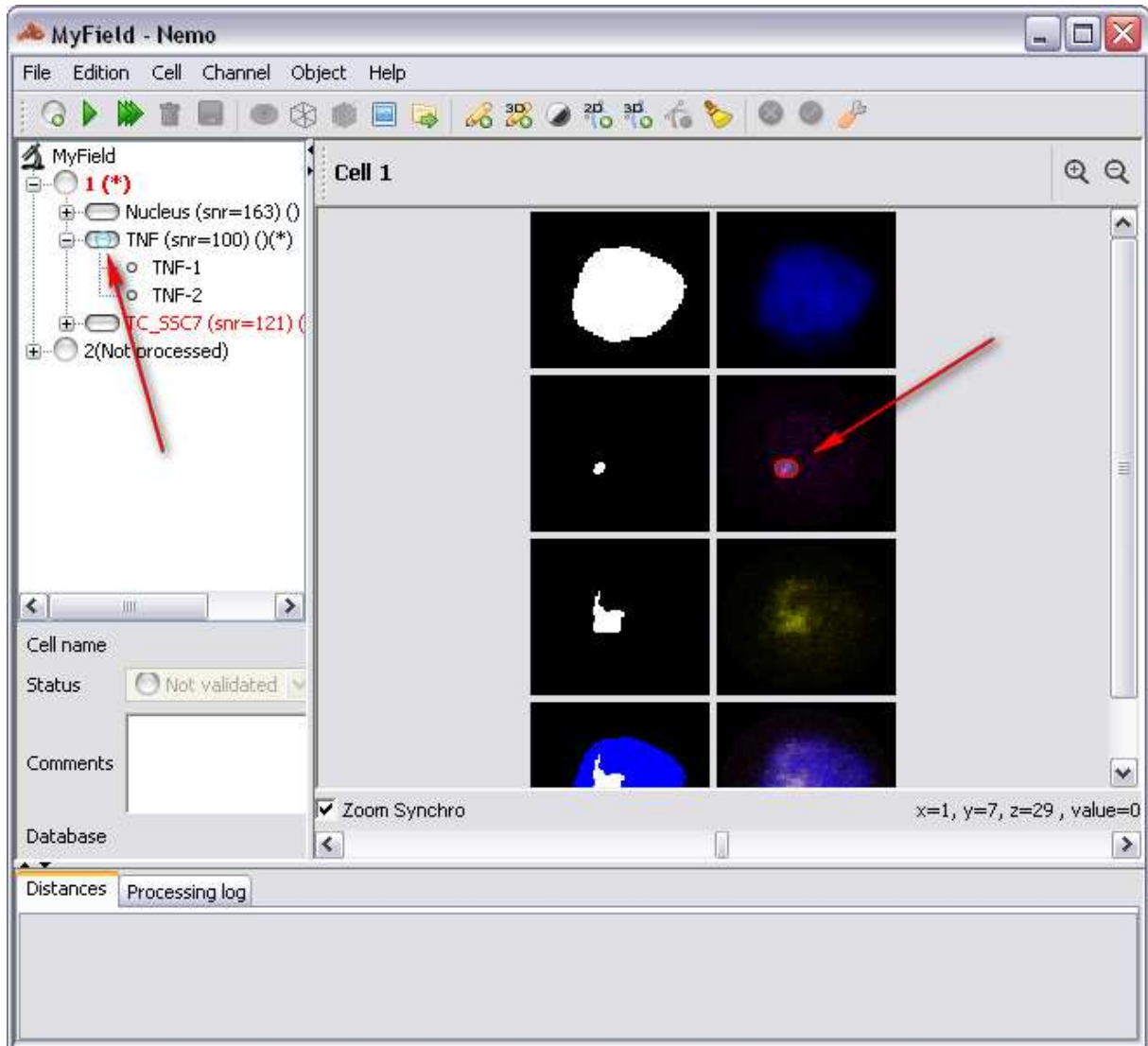
This kind of image mask is defined for all images in channel stack (scroll the Z dimension to see that mask is the same for all slices). At processing time Nemo will replace masked pixels by 0 values. You can also mask all channels with the same mask by selecting the cell to mask in the tree view before using the *Edit mask image tree* view contextual menu.

13.2.2   Edit per slice mask

In some situations, you may want to exclude artifacts on some specific slices only (artifacts between objects in Z dimension but in the same XY area). To edit per slice image masks, select the channel (or cell) and use the tree view *Edit per slice image mask* contextual menu. The following dialog appears:
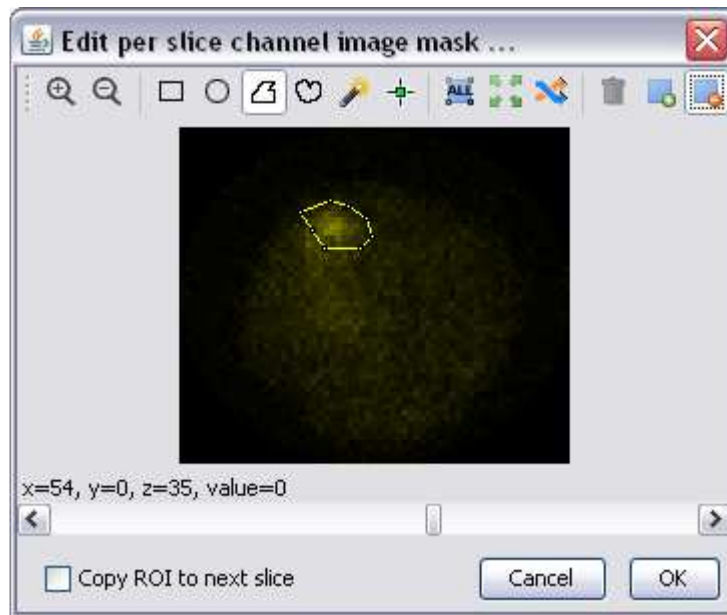
This dialog provides the same function than the previous except that **you can define one different ROI per slice**. Check the *Copy ROI to next slice* checkbox to duplicate current ROI to any slice you browse using the Z scroll bar. When object to mask do not vary too much between slices, user can draw a first ROI (main shape), then duplicate this ROI using the *copy ROI to next slice* function, and finally adjust each slice ROI to image. After clicking on the OK button, Nemo display the defined mask (red border for exclusion, green border for inclusion) in the channel images. A small blue circle will be displayed on the masked channel tree view icon (or cell icon if entire cell was masked). You can check that ROI are different for each slice by browsing image stack.

13.2.3   Remove image mask

Image mask can be removed from channel or from cells by using the tree view contextual menu *Remove image mask*. Since the masking process does not change source images, you will get unchanged raw images.

13.3 Detect object from manual seed

We saw that Nemo use some automatically threshold seed pixels to detect objects by 3D connectivity algorithm. You can ask Nemo to skip the seed pixels detection phase and to use a manually image positioned pixel as seed. This function is useful in situations where tuning threshold bring too much object perturbation and when there is no artifact to mask in raw images. Select the channel you want to add object on and use the *Detect object from manual seed* tree view contextual menu. The following dialog appears:
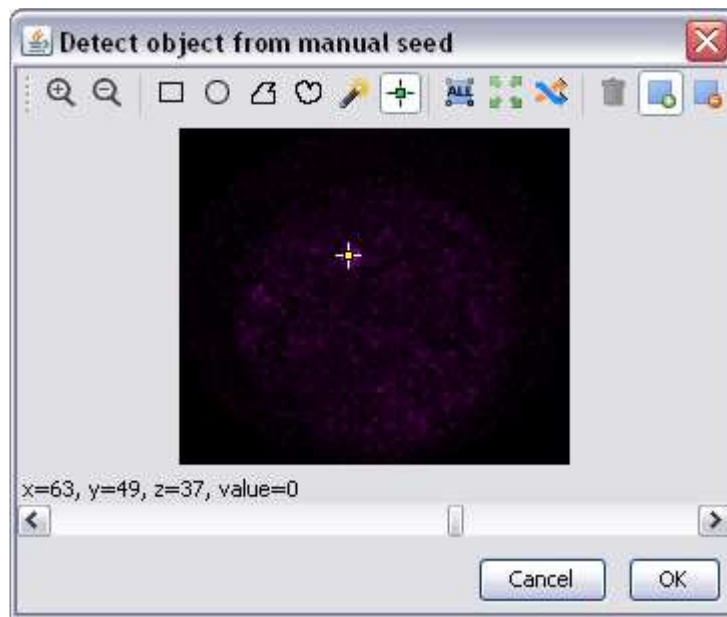

Click on the Point tool icon in the toolbar (⊹) and then click in the image at the place you want to put the seed. Click on the OK button to let Nemo starting the process using your manual seed. After processing, detected object will be displayed in the segmented images, a specific icon (🖋) is used in the tree view to denote objects been detected from manual seed.

13.4 Draw 3D object

If none of previously described techniques gave satisfaction, you can draw object directly on channel stack using successive ROI (one roi per slice). Select the channel you want to add object on and use the *Draw 3D object* tree view contextual menu. The following dialog appears:
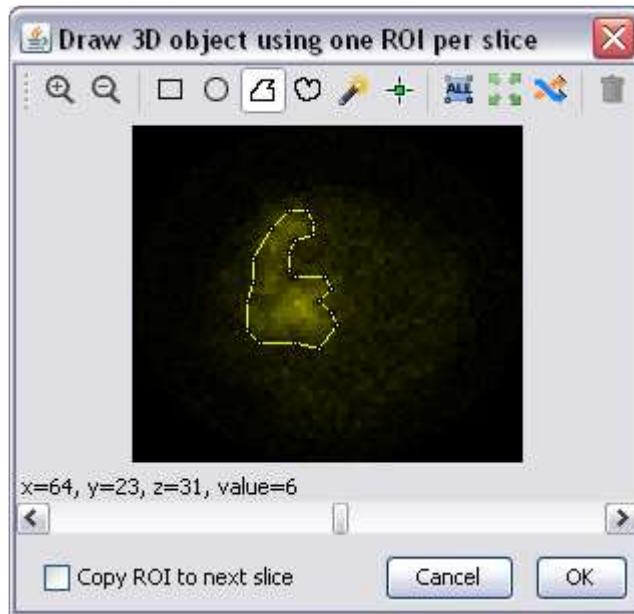
You can use any selection tool to outline object slice per slice, use the *Copy ROI to next slice* checkbox to duplicate current ROI to any slice your browse using bottom scrollbar (Z dimension scrollbar).

NB:
Please, outline only one continuous object using this method, if you define more than one object, Nemo will probably get confused.

14 Resetting channels and cells

You can reset a channel or a cell using the tree view *reset* contextual menu. Resetting a channel will remove all objects, measurements, masks, manual threshold and temporary files (TopHat and median filters are stored as files until mask is untouched or while is no mask).

15 Open in file explorer

Use this menu to directly open the field directory in your file manager, this menu works under windows but can be buggy with Linux (depending on your desktop manager).

16 Show in separate windows

This menu display all channels merged segmented images and raw image.

17 Using the slice cutter

Sometime many fully black slices can appears before and after pertinent images, this can be a problem for automatic threshold because of the histogram shift. You can remove such black slice using the Z cutter: select the cell in the tree view and use the *Z cutter* tree view contextual menu; following dialog appears (use the zoom button to enlarge images).
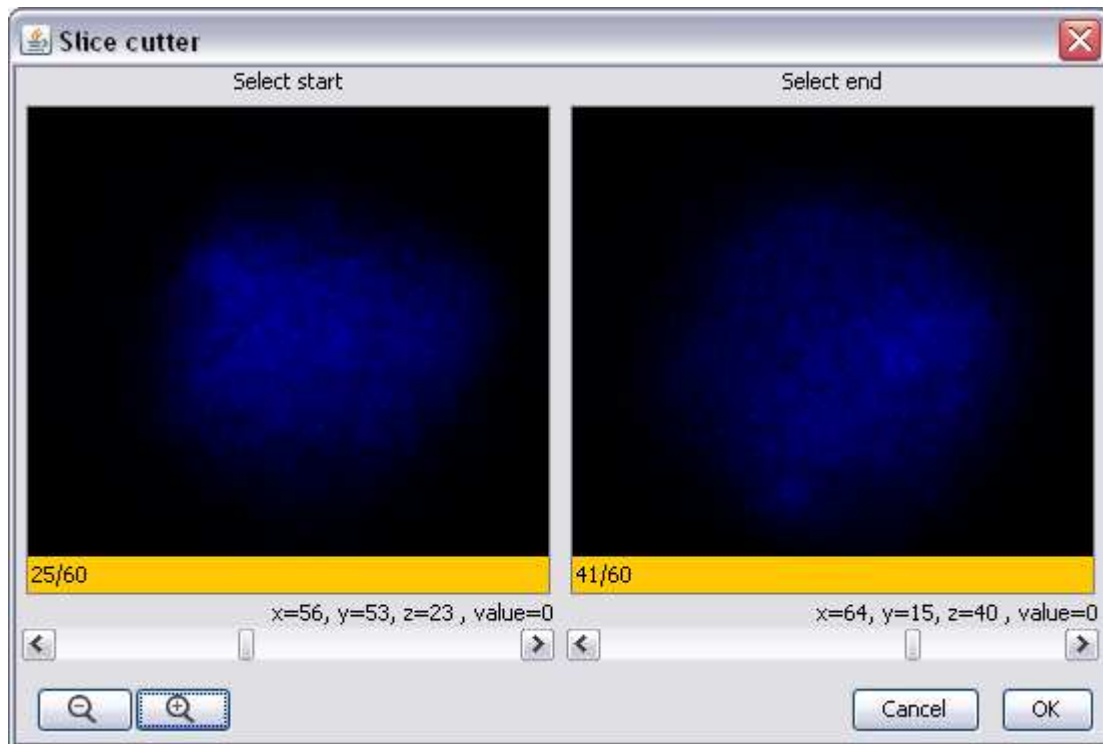
Select the new starting slice by positioning the left scrollbar, and the new ending slice by positioning the right scrollbar. Slices before the new start and after the new end will be physically removed: this is a destructive operation; removed slices cannot be restored later.

18 The 3D projection view

After cell is processed, user can show a Y axis cell projection view displaying all channels.

19 The 3D reconstruction view

After cell is processed, user can show a 3D cell reconstruction view displaying nucleus as transparent shape, chromosome territories (if any) as points cloud and gene as shape. Click on the cell to display in the tree view and choose the *Show 3D reconstruction view* menu contextual menu (or cell menu). The following window appears:
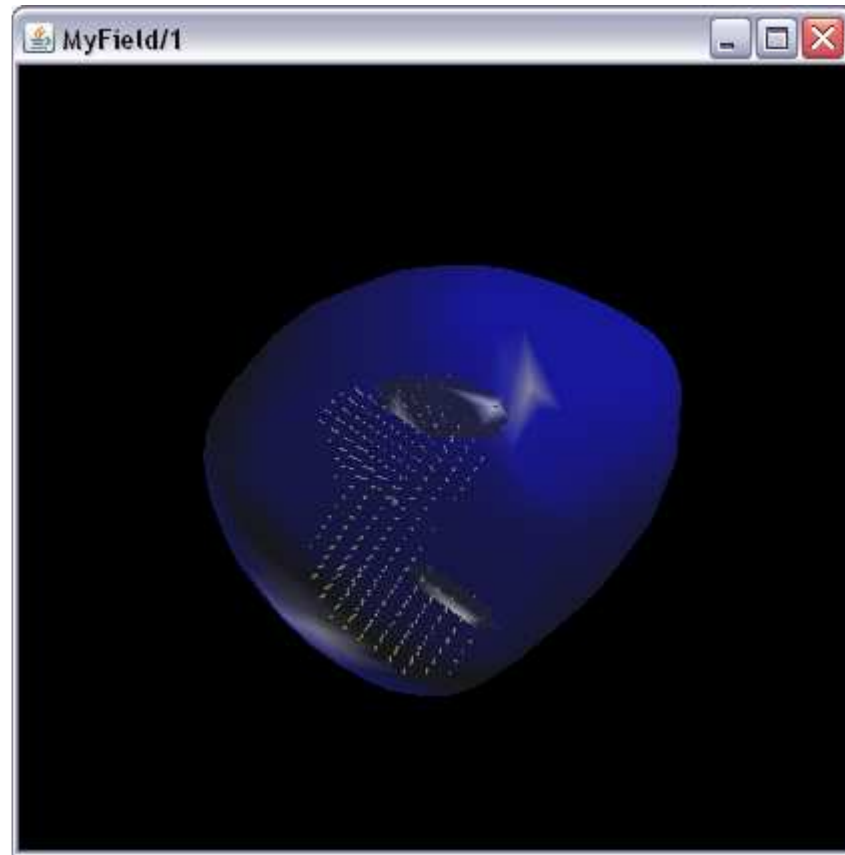
You can rotate cell by using mouse left button, move the cell with the mouse right button and zoom-in /zoom-out with the mouse scroll button.

20 Validating results

After cell has been processed (one shot in automatic mode or through many manual tuning), you can select cell status and write a comment in the cell properties panel.

If database is setup and reachable, any status or comments update will trigger the results save to database. You can also force the results database save by clicking on the save (🖫) toolbar icon or tree view contextual menu.

21 Browsing database

You can browse the results from the MySQL database using the database browser tool; use the *File->Database browser* menu to open the following window.
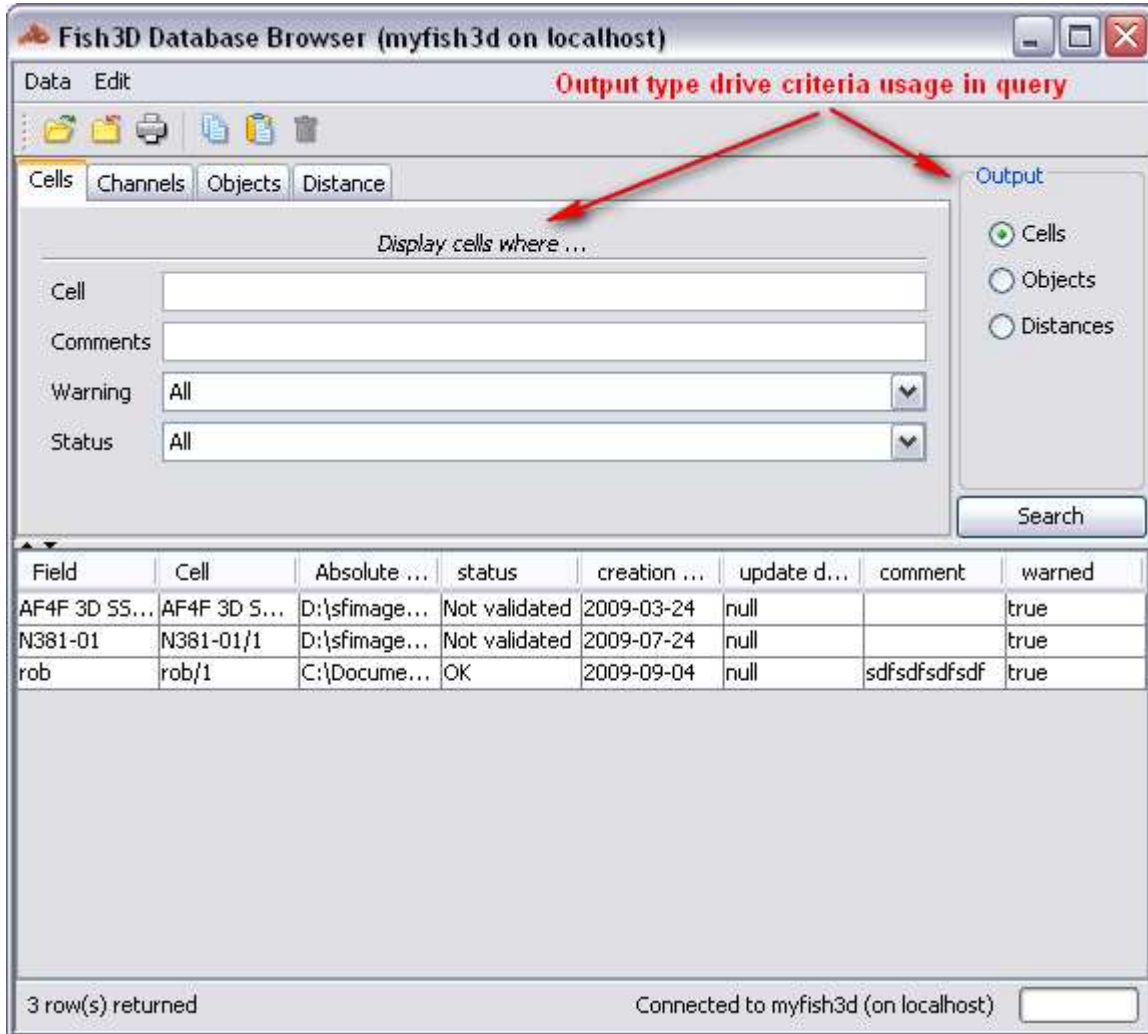
Select the output type in the top right panel (cells, objects or distances), this will change the result table columns composition and the <u>way criteria will be used in query</u>. By example, if output is *cells*, *channels* tab criteria will be used as: *Display cells having at least one channel* …
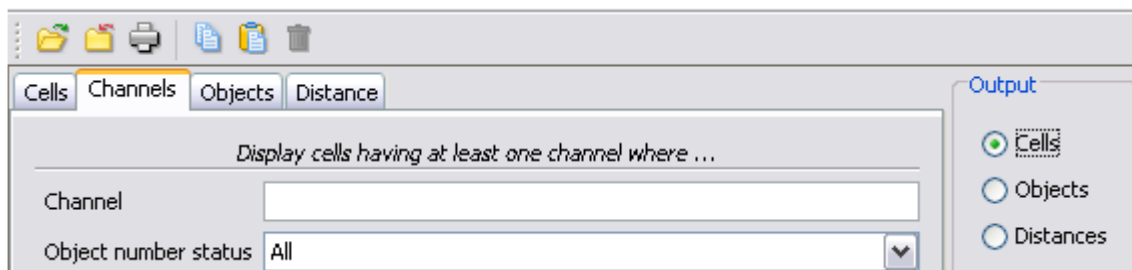
If output type where *Objects*, channels tab criteria were used as: *Display objects from channels where…*
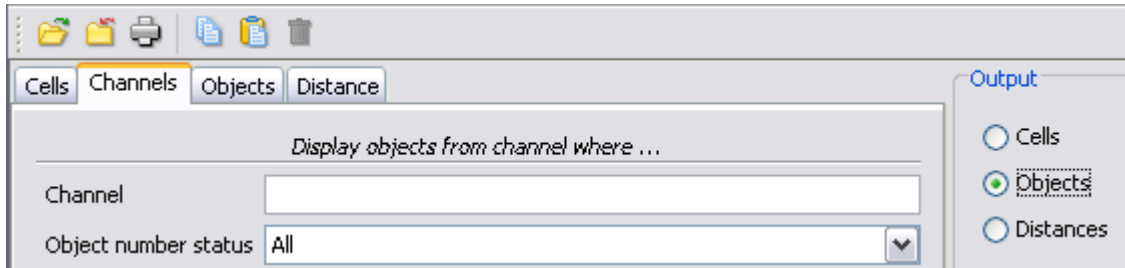
So be careful to read the criteria tab top label to understand how criteria are used. You can use wildcards character:

%   replaces any number of characters
_   replaces only one character

Click on the column headers to change sort order, you can select result table rows using mouse left button and copy (CTRL-C) them into clipboard. Click on the trash to delete cell from database only (cell is not deleted from files so you can still open from field). The print button (🖨) produce a very basic result table output.

22 Using Nemo in command line mode

You can use Nemo in command line mode; this can be useful for batch processing on servers or clusters. Use the following parameters:

```
-c (--cut)                      : Automatically cut cells
 -d (--dirFromConfFile)         : Use configration file to read field path value from
 -f (--file) FILE               : Configuration file to open
 -g (--noGUI)                   : Do not show graphical user interface
 -h (--help)                    : Show this help
 -l (--logFile) FILE            : send logging informations to file path
 -n (--startCutFrom) INT VALUE :   :Start automatic cut from value
 -o (--logToStdout)             : send logging info to stdout instead of nemo.log file
 -p (--processAll)              : Process all cells
 -v (--version)                 : Show version
```

Here is an example of command line to ask Nemo processing a field in command line mode with no graphical user interface and redirecting logging information to stdout. We assume that Nemo.jar file is in the *C:\nemo* directory and that our field configuration file is in the *C:\MyField* directory.

After been positioned into the Nemo directory (*cd C:\Nemo*), type:

.\jre\bin\java -Xms512m -Xmx1024m -jar nemo.jar -f C:\MyField \mfish3d.conf -g -p

This will start field outlined cells processing in command line mode.

23 Licence

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