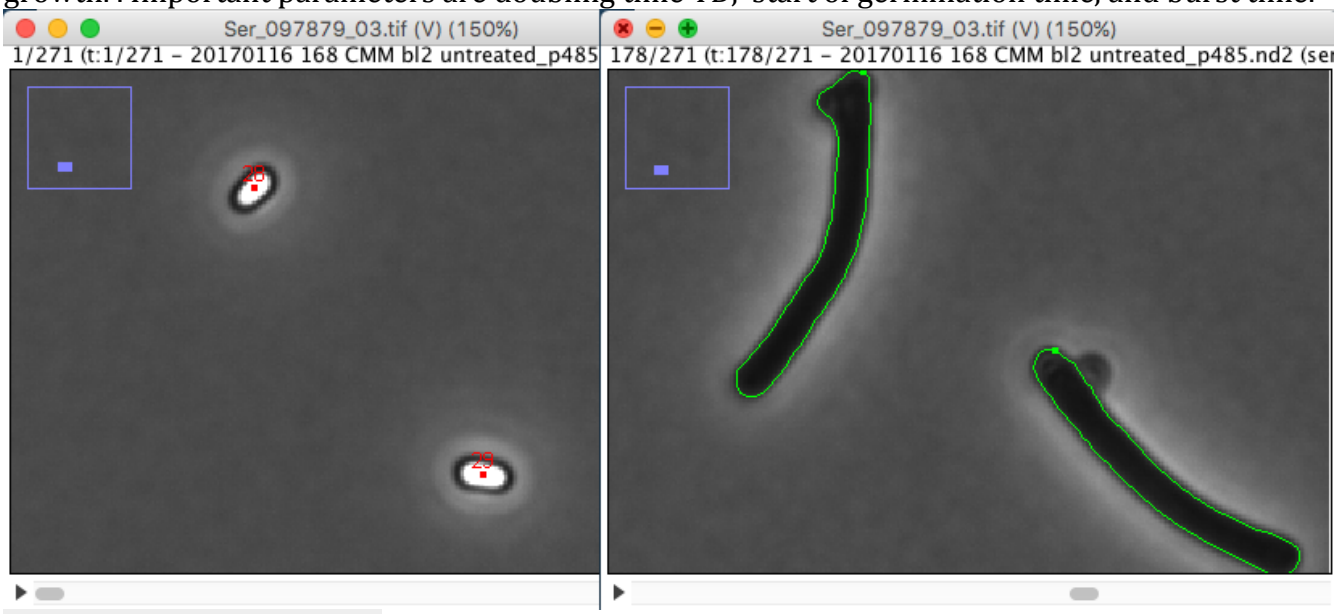
SporeTrackerX

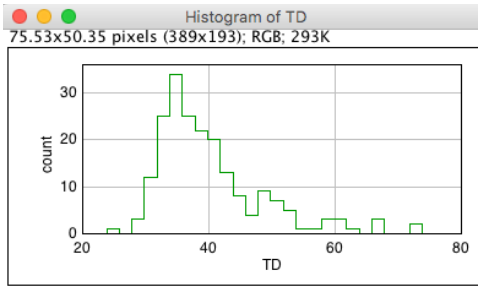
N. Vischer
18-Mar-2018

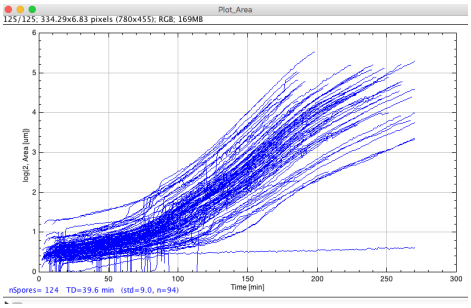
SporeTrackerX is an ImageJ/ObjectJ project for the analysis of the germination process of bacterial spores. It is suitable to handle large data sets (terabytes) on a normal desktop or laptop computer.
This documentation proposes a file structure for a complex set of experiments, explains the conversion of Nikon .nd2 to TIFF files, and describes the analysis of bacterial germination and growth. . Important parameters are doubling time TD, start of germination time, and burst time.

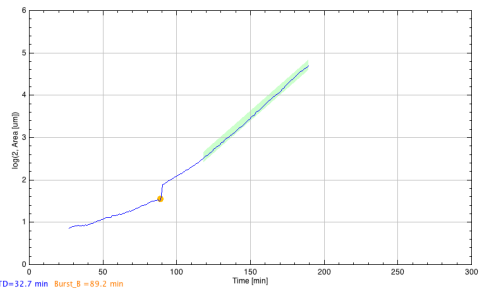


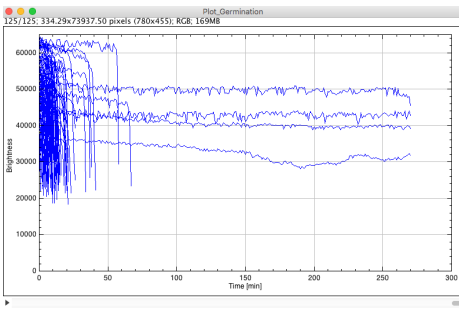
Linked results (0) Unlinked results

[Stat]	TD	GermA	BurstA
Count	16	18	14
Mean	31.0	20.22	112.9
StDev	1.45	9.21	14.95
1			
2	29.2	18.00	104.0
3			
4	30.5	46.00	130.0
5		14.00	96.0
6	29.2	20.00	
7	33.9	15.00	102.0
8	30.5	9.00	117.0
9	29.0	17.00	97.0
10			









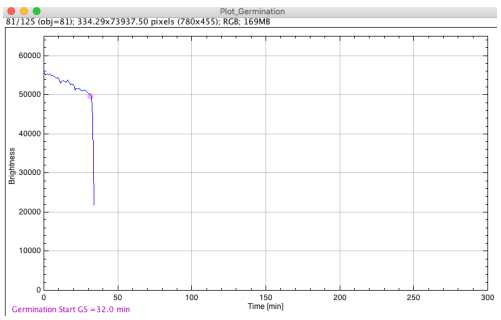


Fig 1: Overview

Data flow

A large number of files was involved during an experiment, which had to be processed on these hardware components:

1. hp workstation + Nikon microscope (acquisition)
2. USB-Storage#1 (5 TB USB3 backup-disk, in locker)
3. USB-Storage#2 (5 TB USB3 analysis-disk)
4. iMac (analysis)

Sequence of user actions:

- On the hp workstation, a suitable (sub-)folder structure was prepared
- Images were acquired and stored as .nd2 files in the predefined locations
- All data were copied to USB-Storage#1
- All data were copied to USB-Storage#2
- Data on the hp workstation were cleared
- USB-Storage#1 was locked as back-up
- USB-Storage#2 was used to convert and analyse images in place.

File organisation

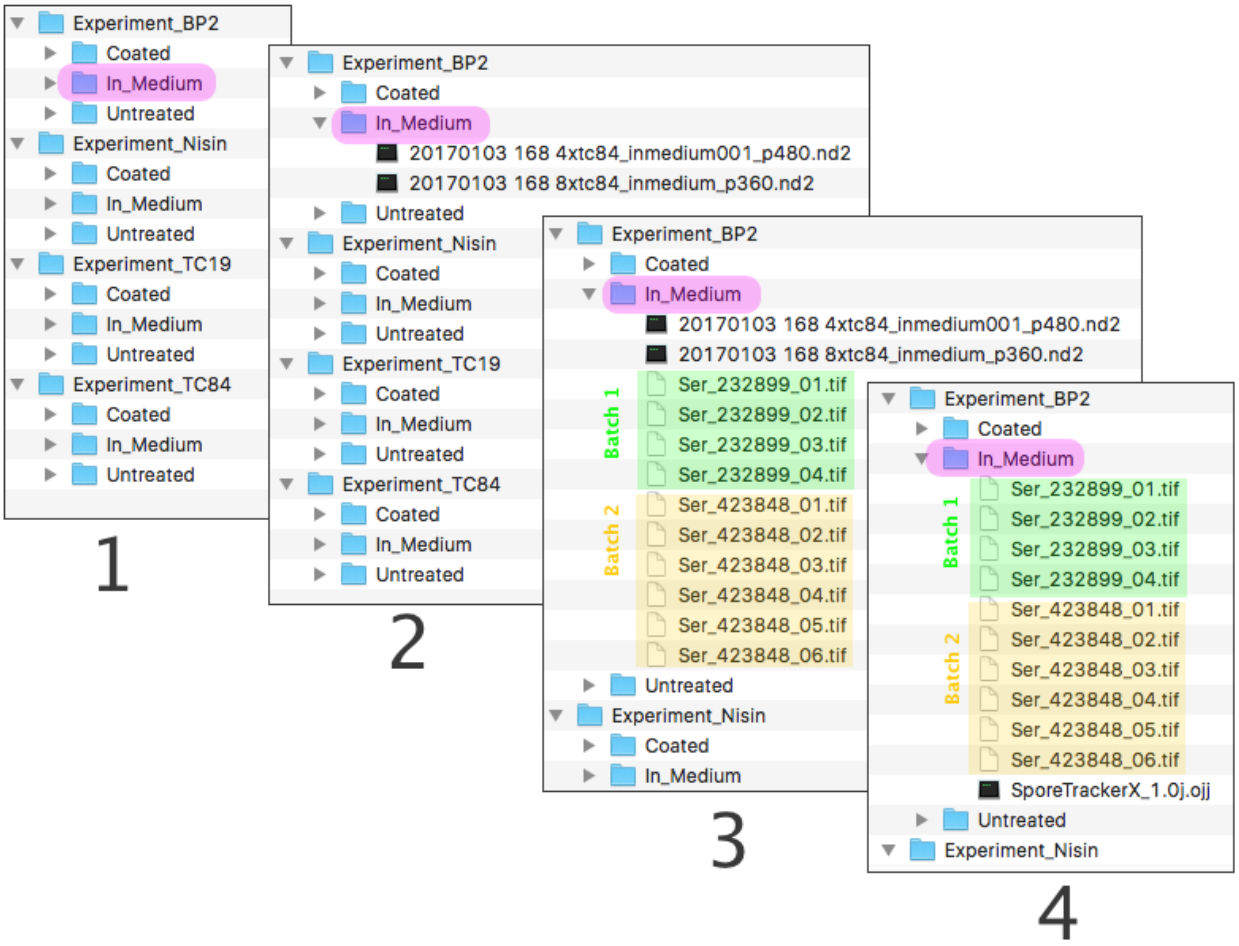


Fig 2: File structure

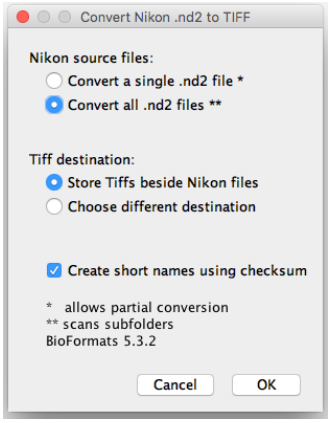
The following steps are suggested to organize the file structure:

Step 1: create empty folders with correct names and hierarchy. In this example, 12 image folders were created.

Step 2: populate all 12 image folders with Nikon .nd2 files. Here, each experiment contains two batches, so we have 24 Nikon files. By now, the optional "preparation time suffix" should be appended: in 'step 2' of Fig 2, "..._p480.nd2" indicates 480 sec between treatment and movie start.

Step 3: Run the macro "Import_ND2_Series_9.txt",
Select:
- "Import All",
- "Store Tiffs beside Nikon files" and
- "Create short file names".

Perform "Tiff-Import" from the top level of all 12 experiments.
You will be guided during the import process. 24 Nikon files will be converted to ~240 tiff movies and stored in the correct locations.
In Fig 2, two different batches with checksums "232899" and "423848" are visible (shaded yellow and green).



Step 4: Add empty copies of SporeTrackerX.ojj to each image folder (12 copies in the example above). For analyzing a set of movies, open the corresponding .ojj file (drag & drop it onto the ImageJ main window), and choose

ObjectJ>Linked Images> Link all images from project folder.
ObjectJ>Save Project
ObjectJ>Show Metadata (optionally, to verify path, short and long names)
ObjectJ>Analyze Spores (takes 10..15 minutes)
ObjectJ>Save Project

As shown in the screenshot, the Nikon .nd2 files are not needed anymore after step 3. The full information is stored in the tiff files. If no error has occured, the Nikon files could now be deleted.

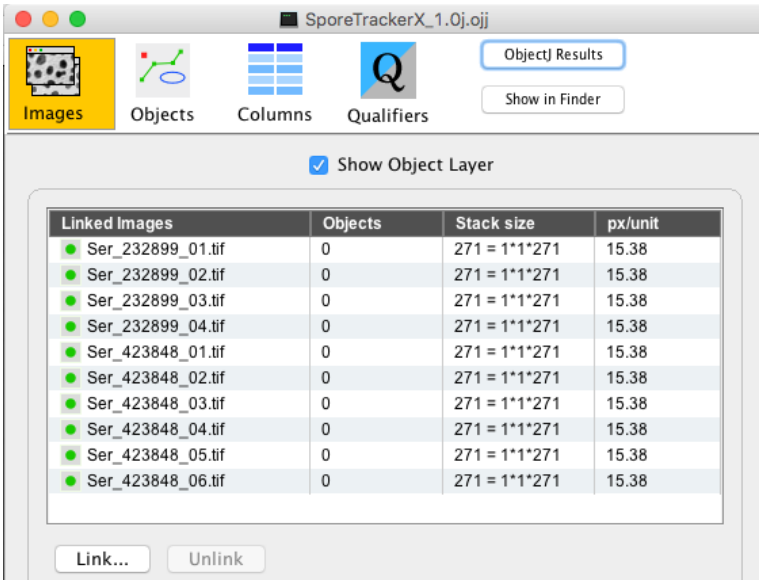


Fig 3: Project window showing linked images

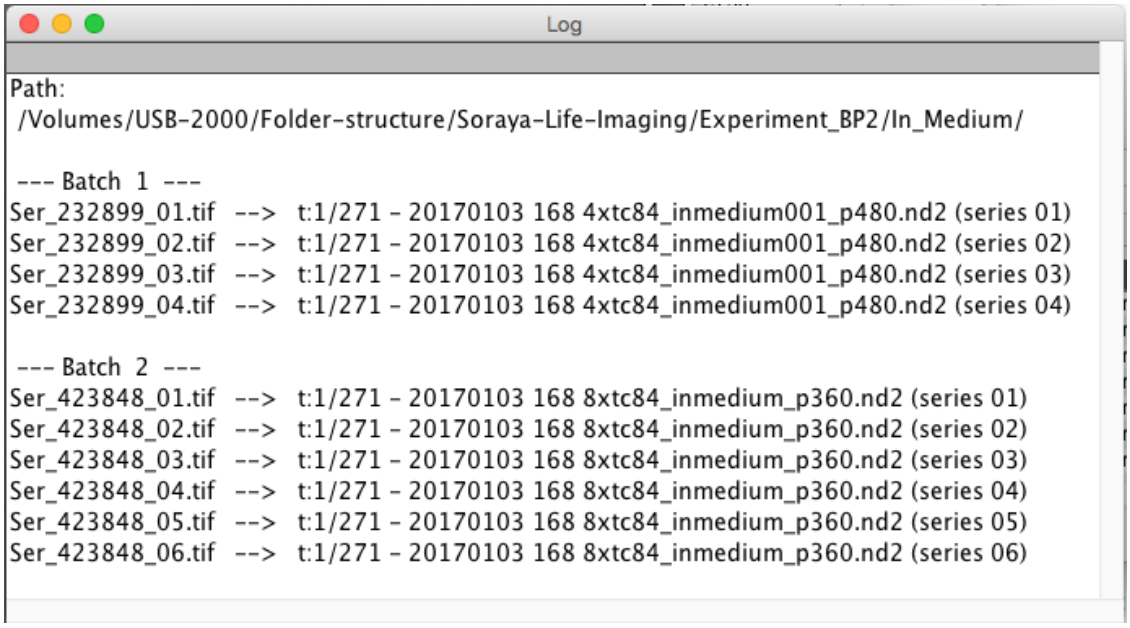


Fig 4: "*ObjectJ>Show Metadata*" shows short and long file names (works also before any spores are marked)

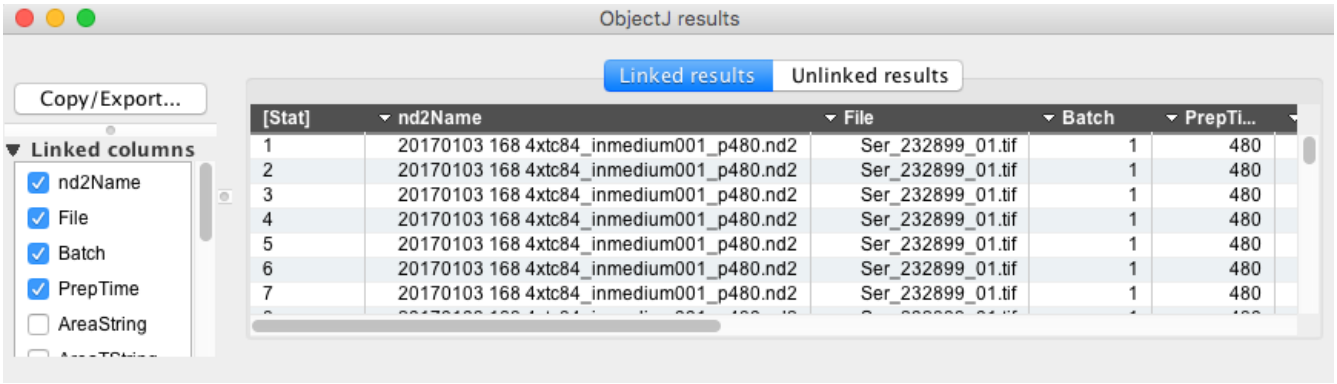


Fig 5. When the spores are marked, *ObjectJ>Show ObjectJ Results* shows long and short names, batch numbers and preparation times. By default, some columns may be hidden (see left panel).

Batches: In this application, the term 'batch' is used for biological repeats. For example, if an experiment is carried out on Monday and repeated on Thursday, we have two batches of movies that reside in the same folder and are statistically combined. Still it is possible to compare the individual batch statistics in case something went wrong.

Disk formats

We use portable USB3 drives (2 TB and 5 TB). These were shipped with NTFS format, but we prefer to re-format them to ExFAT, so that read-write operations are possible on both platforms (MacOS and Windows). We used the iMac for re-fromatting; in one case it appeared that a drive re-fromatted on a Windows computer was not writable on a Mac

> Make sure the computer does not go to sleep half way
(Mac: System Preferences>Energy Saver>Copmuter Sleep)

Table 1 An example of the storage space required for a set of experiments and the estimated processing time.

Storage	
Number of image folders (experiments)	12
Movies per folder	20
Frames per movie	400
MB per frame (16-bit)	8
Total storage for entire set of experiments (1channel)	0.75TB
Processing time	
Copying from USB3 HD to USB3 HD	0.2 min per GB, 3 hrs per TB
Converting .nd2 file to .tiff file on USB3 HD	1 min per GB
Analyze one movie (10 spores, on USB3 HD)	1 min
Analyzing 240 movies (2400 spores on USB3 HD)	4 hrs

-Nikon HP workstation and desktop (iMac) computer

The Nikon microscope stores the acquired images in very large .nd2 files (so far: 20..30GB). As shown above, we import individual movies (e.g. series01, series02) as tiff stacks. The computer must be able to hold an entire movie in RAM. So far, we had 2GB movies which fit easily in the recommended 75% of 8 GB RAM of our iMac. Much larger movies must be imported on the Nikon hp workstation (built-in: 64 GB). Later, for analysis, the movies don't need to fit into RAM as we can work fully virtually.

So far, we followed this procedure:

- copy Nikon files from the Nikon/HP computer to a portable USB3 drive (2 or 5 TB)
- subsequently, the files remain on USB3 drives and are not copied to internal computer disks.
- re-connect the USB3 drive to a local desktop compouter (iMac)
- perform file organisation, Nikon import and analysis as shown above in step 1 to step 4.

Long and short file names

- The "Nikon Import" macro creates short .tif file names that hold a checksum signature derived from the long name. Different batches are therefore easy to distiguish. The long file name may contain the preparation time (treatment bacteria until movie start),

for example:

- *long:* 20170103 168 4xtc84_inmedium001_p480.nd2
- *short:* Ser_232899_01.tif

where *_p480* is the preparation time in seconds, *_232899_* is a unique signature, and, and *_01* is the first of several series (movies) contained in the .nd2 file.

Note: From version 1.0f, ObjectJ has a "Virtual Flag" checkbox in the panel for linked images, which is switched on in SporeTrackerX. This means that double-clicking the name of a linked image, or of a results row, will show the corresponding frame immediately without first loading the entire movie.

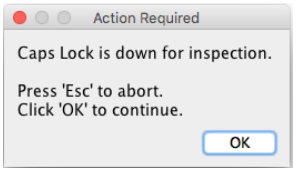
Analysis

When starting the analysis, bright spores with red "Bright" markers until bright-to-dark transition, and then in subsequent time frames the outline of growing filaments is marked with green "Contour" markers. A collision with other filaments will stop the further evaluation. This is repeated for all linked movies. Spores that appear as **close neighbors** in the first time frame are ignored.

- Start Analysis via *ObjectJ>Analyze Spores*
  this may take quite some time
  make sure not to manually interfere, but you can:
  - activate a different application (e.g. click in desktop to activate Finder)
  - choose ImageJ>Hide ImageJ [H] (but don't use the shortcut H)
  - interrupt with Caps Lock (see below).

- During the analysis, a cumulative growth plot will be updated
  after each movie and show basic statistics

Interrupting analysis safely with Caps Lock:
You can safely interrupt the process by setting the Caps Lock key, activate and inspect (but not close) individual windows. Release Caps Lock and click OK to continue analysis.



Stabilisation
In the old version (SporeTracker), spatial stabilisation was necessary before analyzing the movies. In SporeTrackerX, we apply individual particle tracking, so that the stabilisation step can be omitted.

Storage of project file
- After analysis, choose *ObjectJ>Save Project*

The .ojj project file stores image links, macros, markers and results. Data can later be post-processed without opening the linked images.

Plots
Calculation of TD and Burst will automatically take place

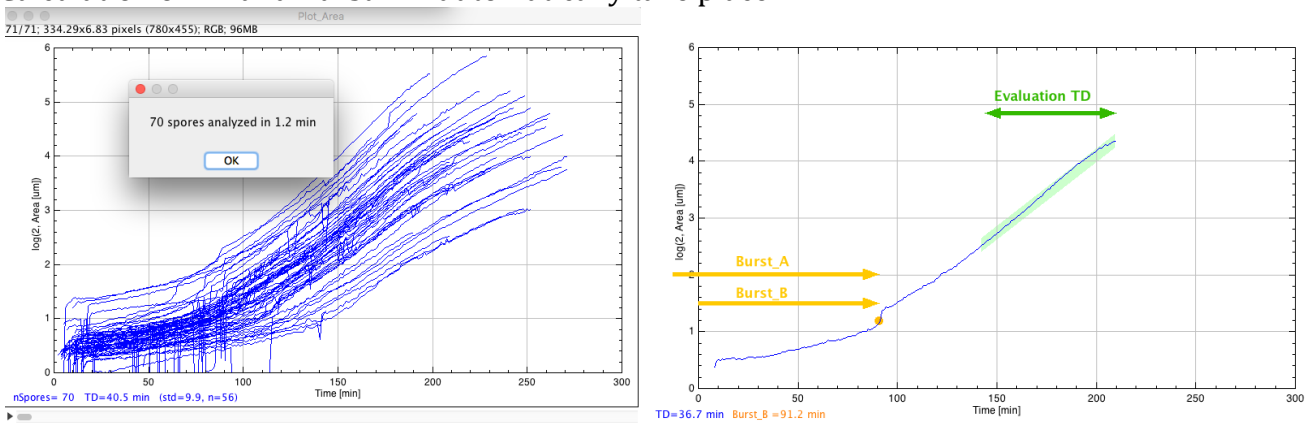


Fig 6: Collective growth plot is updated during analyses (left); single plot shows evaluation slope in green for calculating TD.

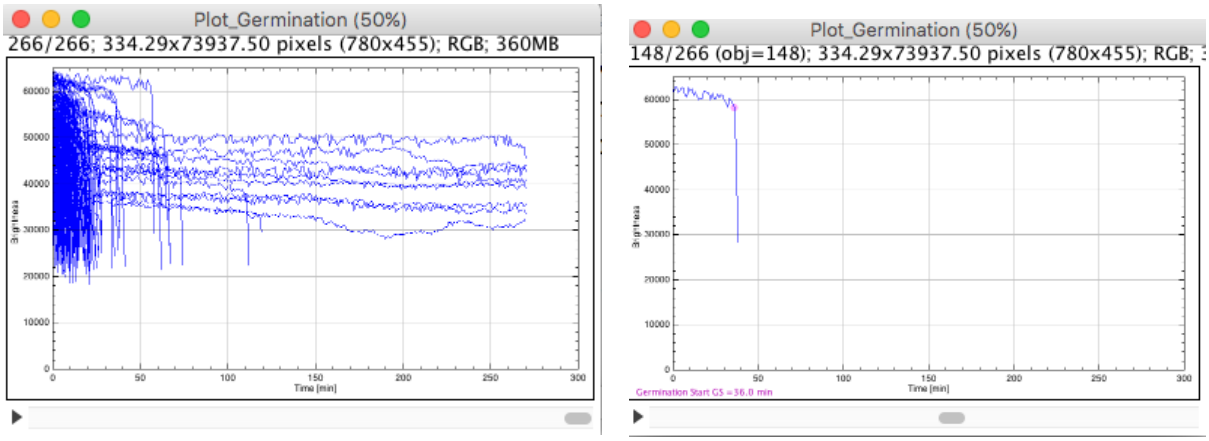


Fig 7: Collective germination plot (left), and individual plot with magenta dot at germination start (right);

Using Plots as Navigation Panel

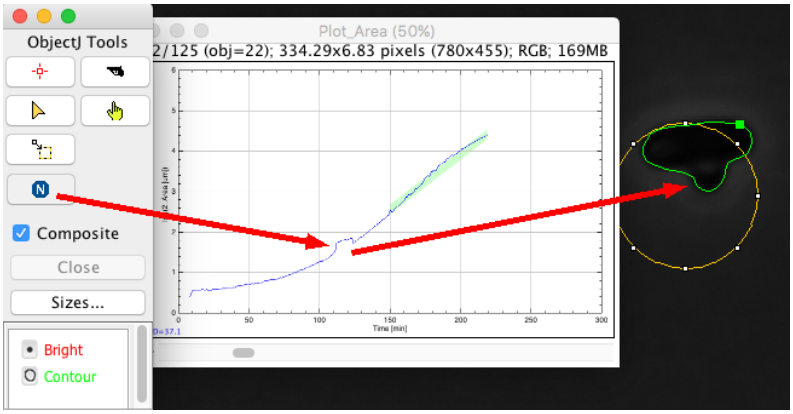
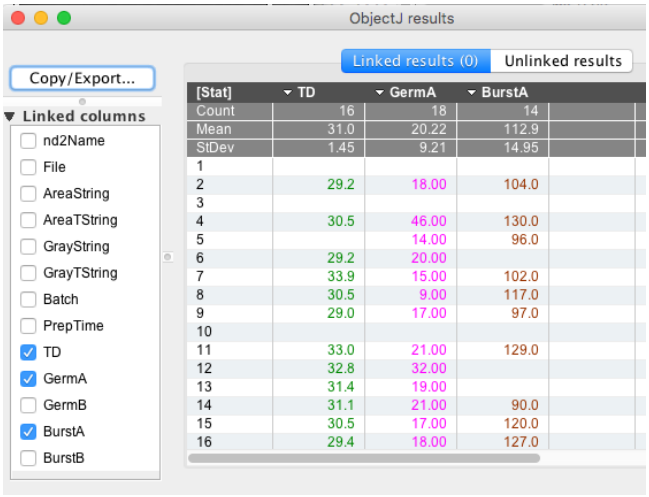


Fig. 8: Select Navigation tool ("N") and drag it across the plot to show cell in corresponding time frame.

Results



[Stat]	TD	GermA	BurstA
Count	16	18	14
Mean	31.0	20.22	112.9
StDev	1.45	9.21	14.95
1			
2	29.2	18.00	104.0
3			
4	30.5	46.00	130.0
5		14.00	96.0
6	29.2	20.00	
7	33.9	15.00	102.0
8	30.5	9.00	117.0
9	29.0	17.00	97.0
10			
11	33.0	21.00	129.0
12	32.8	32.00	
13	31.4	19.00	
14	31.1	21.00	90.0
15	30.5	17.00	120.0
16	29.4	18.00	127.0

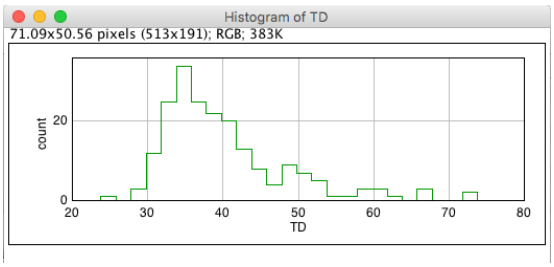


Fig 9: Linked results and histogram

ObjectJ Results:
TD Doubling time (minutes)

GermA Germination start (measured from cell treatment)
GermB Germination start (measured from movie start)

BurstA (measured from cell treatment)
BurstB (measured from movie start)

Difference between A and B, here: 5 minutes, is the preparation time

Histograms
Right-click a column title and choose "Show Histogram" to create a histogram. Click "Modify" to change color bin width and range. Histograms shows "all" and "qualified" in the same plot.

Qualifying
If a growth-plot window is open, you can disqualify the corresponding cell by choosing *ObjectJ>Toggle Qualification [Q].* This will toggle the value in column "Q" between 1 to 0, and update qualification of all other objects as well. Disqualifying make the plot area gray, and qualifying makes it white. These values are stored in the .ojj project file.
-To update qualification after loading the project file, choose *ObjectJ> Qualification...* Here, you optionally can kill unqualified objects.

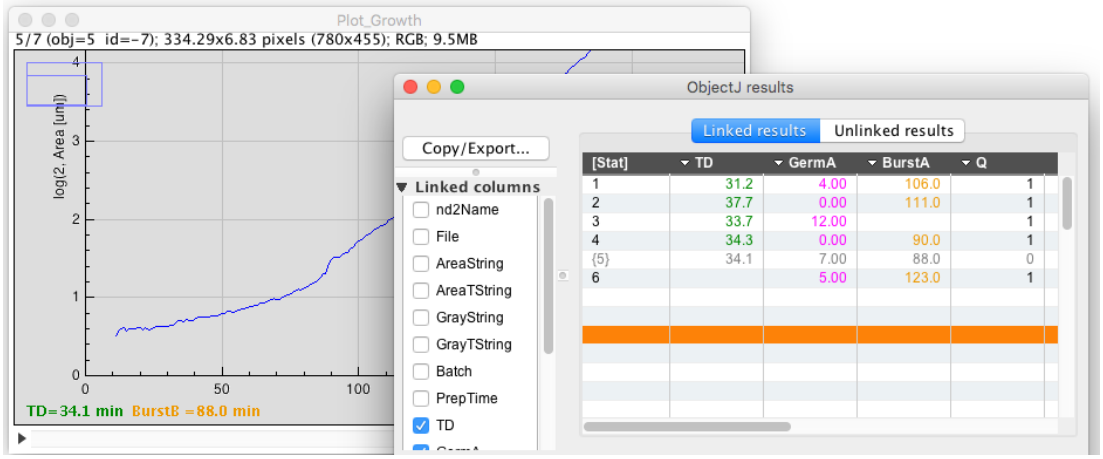


Fig. 10: Object (#5) was disqualified with key "Q", i.e. grayed and marked as Q=0 in the results.

Burst
The burst time can be set manually: locate the cursor in the growth plot window and press ***key B***, The brown cicular (=automatic) burst sign will turn into a square (=manual) burst sign at cursor position, and the value is entered in column BurstM. Press ***key B*** again on the same location, and the sign will be removed.

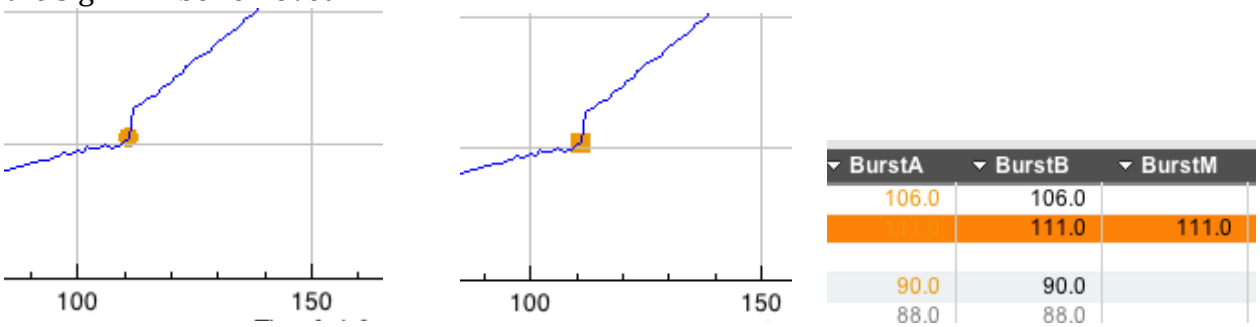
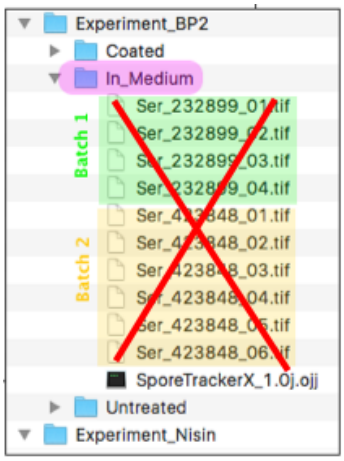


Fig. 11: Automatic burst (left) was marked manually (right) with key B. The value is soted in BurstM (M= manual).

ShadowDirectory for Backup

For backing-up, it is not practical to duplicate all the gigabyte-movies which usually were not changed. Instead, you can back-up only the .ojj project files that contain the analysis: create a "ShadowDirectory", which is a copy of the entire directory tree, but containing only files with the .ojj extension.
From the experiment in Fig 2, a shadow directory would contain 16 folders, 12 of which are "image folders" containing a total of 12 .ojj project files, but no other file types. The shadow directory will fit easily on a USB stick or on a Dropbox directory. See:
https://sils.fnwi.uva.nl/bcb/objectj/examples/utils/ShadowDirectory



Handling the .ojj project file in absence of the movies

The very large movie files cannot easily be copied from A to B, or e.g. to the DropBox. However, many useful things can be done with an isolated .ojj project file. Here is a list of possible(+) and impossible(-) actions when the project file is not located in the folder with linked images:

+ create growth plots
+ create germination plots
+ create histograms from ObjectJ results
+ observe and export ObjectJ results table
+ qualify, disqualify or delete objects
+ add/change burst time
+ unlink images
+ alter and save the .ojj project file
- observe images and contours
- use plot to browse through movies
- link images

ObjectJ Menu:

Analyze Spores

Existing spore markers (if any) will be deleted after confirmation. Then bright and dark particles will be detected throughout the movie and identified as growing objects. An object consist of a red "Bright" marker in frame#1, and any number of green "Contour" markers in subsequent time

frames. The number label appears in frame#1. Per spore object, the growing area versus time is recorded and stored as string in Objectj column "AreaPairs". Such a string consists of value pairs separated by semicolon, and each pair is consists of a time and area value separated by a space. This same technique is used for "GrayPairs" to quantify germination (bright to dark transition). After analysis, "Plot_Growth" and "Plot_Germination" are created from the corresponding columns.

Plot Growth and Germination
"Plot_Growth" and "Plot_Germination" are created from the corresponding columns. A plot window is a stack holding one plot per object. One additional slice is appended to the stack containing a superimposition of all plots. Typically, a plot window needs not toe be saved, as it can be rebuilt at any time.

Open All Virually
Opens all linked images as virual stacks, i.e. only a single frame is loaded into RAM. The large stacks should be virutally open so that many operations such as double-clicking a "linked image" or a "linked result" does not loose time loading the entire stack.

Toggle Q flag
Qualifies/disqualifies the object shown in the current growth plot, sets the plot background to white/ gray, and sets the value in column "Q" to one/zero.

Qualification...
(Dis-)qualifies all objects depending on value in column "Q", and provides the option to kill unqualified objects (which will re-draw the plot windows).

Show Metadata
Shows project path, long and short file names, and batch number. Long file names derived from the label in the stack's metadata.

Set Sign Burst
Use this by activating the Plot_Growth window, position the cursor upon the time where the burst should be marked, and press the shortcut key "B". Any old burst marking will be replaced by the new one, or removed if key was pressed twice on same position.

Set Sign FirstDiv [F]
Similar to "Sign Burst"

Set Sign T1 [1]
Similar to "Sign Burst". Sets the starting point of the time window to calculate TD.

Set Sign T2 [2]
Similar to "Sign Burst". Sets the end point of the time window to calculate TD.

Activate Navigation [N]
Arranges windows "Plot_Growth" and "Plot_Germination" on the right hand of the screen, and activates the navigation tool in ObjectJ Tools ("N" icon). If an object is selected (e.g. clicked in the ObjectJ results row), the Navigate command activates the plot of this object in both plot stacks. As the "N" tool is active, the user can now click and drag inside the plot and observe the movie synchronously.