Video Article

Quantitative Live Cell Fluorescence-microscopy Analysis of Fission Yeast

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Abstract

Several microscopy techniques are available today that can detect a specific protein within the cell. During the last decade live cell imaging using fluorochromes like Green Fluorescent Protein (GFP) directly attached to the protein of interest has become increasingly popular [1]. Using GFP and similar fluorochromes the subcellular localisations and movements of proteins can be detected in a fluorescent microscope. Moreover, also the subnuclear localisation of a certain region of a chromosome can be studied using this technique. GFP is fused to the Lac Repressor protein (LacR) and ectopically expressed in the cell where tandem repeats of the *lacO* sequence has been inserted into the region of interest on the chromosome[2]. The LacR-GFP will bind to the *lacO* repeats and that area of the genome will be visible as a green dot in the fluorescence microscope. Yeast is especially suited for this type of manipulation since homologous recombination is very efficient and thereby enables targeted integration of the *lacO* repeats and engineered fusion proteins with GFP [3]. Here we describe a quantitative method for live cell analysis of fission yeast. Additional protocols for live cell analysis of fission yeast can be found, for example on how to make a movie of the meiotic chromosomal behaviour [4]. In this particular experiment we focus on subnuclear organisation and how it is affected during gene induction. We have labelled a gene cluster, named Chr1, by the introduction of *lacO* binding sites in the vicinity of the genes. The gene cluster is enriched for genes that are induced early during nitrogen starvation of fission yeast [5]. In the strain the nuclear membrane (NM) is labelled by the attachment of mCherry to the NM protein Cut11 giving rise to a red fluorescent signal. The Spindle Pole body (SPB) compound Sid4 is fused to Red Fluorescent Protein (Sid4-mRFP) [6]. In vegetatively growing yeast cells the centromeres are always attached to the SPB that is embedded in the NM [7]. The SPB is identified as a large round structure in the NM. By imaging before and 20 minutes after depletion of the nitrogen source we can determine the distance between the gene cluster (GFP) and the NM/SPB. The mean or median distances before and after nitrogen depletion are compared and we can thus quantify whether or not there is a shift in subcellular localisation of the gene cluster after nitrogen depletion.

Video Link

The video component of this article can be found at http://www.jove.com/video/3454/

Protocol

1. Fission yeast culture

1. Prepare Edinburgh Minimal Media (EMM) and EMM without ammoniumchloride (EMM-N) [8]. To reduce autofluorescence the glucose solution should not be autoclaved but instead filter sterilised using a 0.2 µm filter and subsequently added to the autoclaved media.
2. Inoculate fission yeast cells freshly grown on an agar plate with rich media, YEA [8], in 3 mL of EMM liquid media with filter sterilised glucose. Use 13mL tubes with a lightly pushed on cap to ensure good ventilation of the cells. Let the cells grow by shaking them with 225 rpm in 30 °C. Keep the cells growing in log phase (1 X 10^6 - 2 X 10^7 cells / mL) for 2 days by counting them using a Burker chamber followed by appropriate dilution, every morning and evening.
3. On the day of the experiment make sure to have cells in early log phase, 5 X 10^6 - 1 X 10^7 cells / mL.
4. To starve the cells for nitrogen during 20 minutes switch from EMM media to EMM-N media. This is done by harvesting 3 ml of cells in a 1.5 mL Eppendorf tube in a bench top centrifuge at maximum 1.5 rcf as centrifugation at a faster speed might induce a stress response in fission yeast [9]. Use the double spinning technique, meaning; first spin for 2 min, then turn the Eppendorf tube 180° and spin again for 1.5 rcf 2 min [10]. This helps to collect all the cells in one pellet at the bottom of the tube. Wash once with EMM-N and then dissolve the pellet in EMM-N and incubate the cells for 15 minutes at 30 °C shaking at 225 rpm and then continue to point 2. Sample preparation.

2. Sample preparation

1. Harvest 1.5 mL of cells in a 1.5 mL Eppendorf tube in a bench top centrifuge at maximum 1.5 rcf as centrifugation at a faster speed might induce a stress response in fission yeast [9]. Use the double spinning technique, meaning; first spin for 2 min, then turn the Eppendorf tube 180° and spin again for 1.5 rcf for 2 min [10]. This helps to collect all the cells in one pellet at the bottom of the tube.

2. Remove the supernatant, but leave 10-15 µL and resuspend the cells in the remaining media. Alternatively add 10 µL of fresh media to resuspend the cell pellet.
3. Make sure to have clear glass slides and cover glass (No 1.5). Normally you do not have to clean them, but make sure they are not full of dust.
4. Take out from the freezer an aliquot of a stock solution of 1mg/mL lectin that has been filter sterilised. The lectin solution can be refrozen a few times. The lectin is used to fix the yeast cells at the cover glass.
5. Place 5 µL of EMM with filter sterilised glucose on the objective glass.
6. Place 5 µL of lectin solution in the corner of the cover glass. Subsequently place 5 µL of cell culture in the same corner, i.e. in the lectin drop. Mix by pipetting a few times and then spread the cell culture-lectin mix throughout the cover glass by using the long side of the pipette tip. Depending on the density of your cell mixture leave everything or suck up excess liquid in the opposite corner of the cover glass.
7. Place the cover glass, top up, with one side on the objective glass and the other side on the bench. Let the cover glass dry a little for a few minutes. It should absolutely not be completely dry, but it should not be too much liquid on the glass.
8. Place the cover glass with an approximate 70° angel from the objective glass by the drop of EMM. Let go of the cover glass so that it will fall top down into the drop of EMM.
9. To seal the edges with silicon grease prepare a 2 mL syringe. Cut the wide end of a 200 µl tip and attach it to the syringe. Fill the syringe with approximately 1 mL of silicon grease. A fine line of silicon is applied to the edges of the cover glass by gently pushing the piston of the syringe. Now you have a small growth chamber of *S. pombe* cells.

3. Microscopy

1. Initialise the fluorescence microscopy by turning on the mercury/xenon lamp, the microscope and the computer. Place the yeast growth chamber in the fluorescence microscope. Use a 63 X objective or a 100 X objective with NA=1.3 or higher. If an oil objective is used, add oil.
2. Use the bright field to find the cells and get a sharp picture.
3. The settings for the fluorescence microscopy vary depending on the fluorochromes used to label the yeast cells and the microscope. We use a confocal microscope Zeiss LSM 700 Laser Scanning Microscope with Plan-Apochromat 63x oil objective lens (NA=1.4) with the 16-line average plane scan setting. The pinhole should be set to 1-1.1 Airy units, which gives an optical slice of 0.8 mm. We detect GFP in Track 1 using the filter set for Alexa 488 with a beam splitter at 582 nm, thus detecting wavelengths between 488 and 582 nm. In Track 2 we use a filter set optimal for mCherry using a beam splitter at 578 nm, thus detecting wavelengths between 578 and 600 nm. This means that in Track 2 both the mRFP (SPB) and NM (mCherry) will be detected.
4. Take as many pictures needed to be able to measure in 60 different cells for each strain. Usually 15 pictures of independent microscopy fields are enough. It is recommended that a new growth chamber with fresh cells is made if your microscopy time exceeds 60 minutes.

4. Quantitative measurement of subcellular distances

1. Open the pictures in the Zeiss Zen Light Edition program. Using the measurements tool measure the distance in µm between the different fluorochromes in all the cells where all the signals are in the same focal plane. Adjust the light intensity and contrast to identify the centre of the fluorescent signal. This simplified protocol uses only two different colours and hence the SPB and NM are in the same channel. The SPB is singled out by its large round structure in the NM. Transfer the distances to a notepad sheet. Measure in at least 60 cells. Other programs such as ImageJ could also be used to measure the distance, but Zeiss Zen Light is preferred due to its higher resolution of the picture and the ease to zoom in and out using that program. Pictures in lsm format opened in ImageJ will have the two channels on top of each other. To make a picture with both channels, first split the two channels and thereafter merge them. Thereafter the line tool can be used to measure the distances as described above.
2. Compare the mean or median subcellular distances between different strains and treatments using a statistical program, for example *t*-test or Mann-Whitney Rank Sum Test, for example by using the SigmaStat-3.5 software. Frequently the data is not normally distributed since there will be a selection for cells with shorter distances between the fluorescent signals since all the signals need to be in the same focal plane to be measured. A *t*-test can only be used when the data has a normal distribution while a Mann-Whitney Rank Sum Test allows the comparison of data sets that lack normal distribution. In a *t*-test the mean of two different datasets are compared while in a Mann-Whitney Rank Sum Test the median is compared.

5. Representative Results

Strain PJ1185:(*h*$^+$ *his7*$^+$::*dis1placR-GFP* Chr1[::*ura4*$^+$ *hphMX6 lacO*] *sid4-mRFP*::*kanMX6 cut11-mCherry*::*natMX6 ura4-D18 leu1-32 ade6-DN/N*) was grown in EMM. A sample was withdrawn and mounted in a small growth chamber and pictures were taken (Fig. 1A +N). Subsequently the growth media was replaced with EMM w/o ammoniumchloride (EMM-N), and cells were grown for 15 minutes while shaking. The nitrogen starved cells were then mounted in a growth chamber with EMM-N and pictures were taken (Fig. 1A -N). The measuring tool was used to measure the distance between the locus (GFP) and the SPB (Fig. 1B and Table 1). In addition, the distance between the locus (GFP) and the NM was measured (Fig. 1B and Table 2). The median subcellular distances before and after nitrogen depletion were compared using the SigmaStat-3.5 software (Table 3). There was a statistically significant shift in the localisation of the gene cluster moving away from the NM towards the SBP. The data measuring the distance between the GFP and the SBP had a normal distribution and hence the mean distances (1.777 µm +N and 1,587 µm -N) could be compared using a *t*-test (Table 1 and 3). There was a significant difference between the two mean values ($P = 0.008$, *t*-test). The data measuring the distance between the GFP and NM did not have a normal distribution and hence the median distances (0 µm +N and 0.390 µm -N) were compared using a Mann-Whitney Rank Sum test (Table 2 and 3). There was a significant difference between the two median values ($P < 0.001$, Mann-Whitney Rank Sum test).

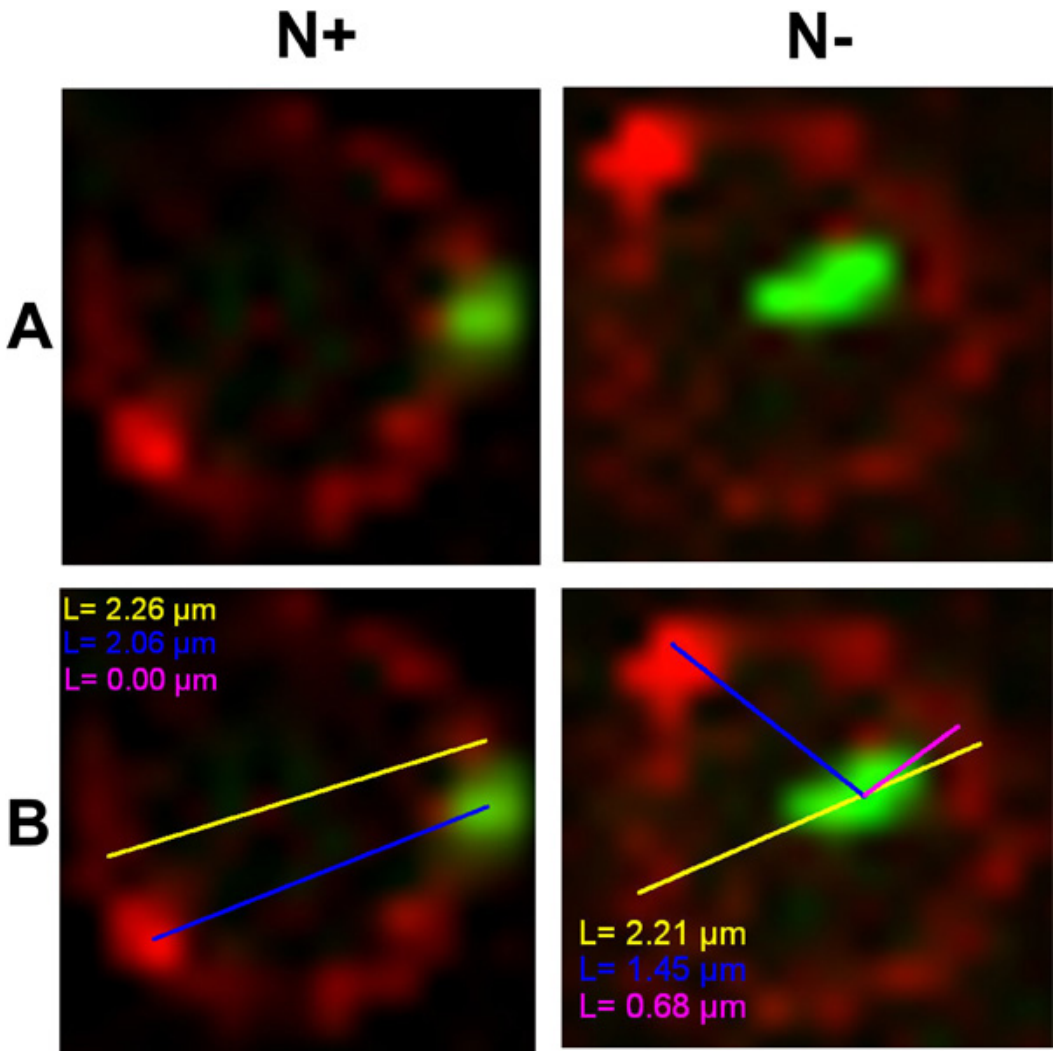

Figure 1. The localisation of a cluster of genes named Chr1, marked by GFP, changed after the nitrogen starvation. (A) Left column, +N, a representative cell nucleus from a cell grown in EMM right column, -N, a representative cell nucleus from a cell grown in EMM-N. Green is the GFP signal labelling the Chr1 cluster, red is the mRFP and mCherry labelling the SPB and NM, respectively. (B) Same cell nucleus as (A) but now with measured subnuclear distances; yellow: the diameter of the cell nucleus, blue: the distance between SPB and GFP signal and pink: the distance between the GFP signal and the nuclear membrane.

d	PJ 1185 N+ GFP-SPB	GFP-NM
2,22	1,69	0,00
2,16	2,12	0,11
2,39	1,47	0,65
2,18	1,45	0,00
2,29	2,45	0,00
2,22	1,70	0,52
2,19	2,11	0,00
2,40	0,96	0,32
2,22	1,73	0,00
2,19	1,35	0,00
2,14	1,81	0,00
2,18	1,92	0,00
2,27	2,17	0,00
2,24	1,54	0,00
2,20	1,75	0,00
2,28	2,11	0,00
2,32	1,38	0,55
2,22	1,99	0,13
2,32	1,10	0,31
2,28	1,50	0,00
2,38	2,46	0,00
2,13	0,93	0,51
2,14	2,10	0,00
2,32	1,95	0,33
2,22	0,56	0,57
2,15	1,19	0,00
2,12	1,55	0,00
2,26	1,30	0,24
2,26	1,62	0,00
2,26	2,27	0,00
2,28	0,87	0,48
2,16	1,76	0,00
2,12	2,19	0,00
2,25	1,90	0,21
2,17	0,64	0,00
2,14	0,70	0,29
2,32	1,96	0,31
2,11	1,80	0,13
2,33	1,94	0,00
2,10	2,03	0,00
2,40	2,06	0,29
2,26	2,06	0,00
2,15	1,92	0,28
2,23	2,21	0,11
2,31	1,84	0,26
2,29	2,04	0,00
2,12	2,00	0,00
2,35	2,43	0,00
2,38	2,20	0,28
2,21	2,13	0,00
2,26	2,10	0,17
2,20	1,94	0,00
2,26	2,23	0,00
2,15	1,84	0,37
2,14	2,15	0,00
2,26	1,08	0,00
2,13	1,91	0,00
2,37	0,85	0,00
2,36	2,14	0,00
2,15	1,64	0,35
2,35	1,94	0,00
2,15	1,45	0,00
2,19	2,25	0,00
2,36	2,31	0,00
2,36	2,48	0,00
2,15	1,98	0,00
2,19	1,45	0,18
2,28	2,00	0,00
2,33	1,94	0,00

Table 1. The measured subnuclear distances in µm of the PJ1185 strain grown in EMM. First row, diameter of the cell (d), second row, distance between the GFP and the SPB, third row, distance between the GFP and the NM.

d	PJ 1185 N-GFP-SPB	GFP-NM
2,21	1,89	0,25
2,26	1,16	0,70
2,18	1,54	0,61
2,14	1,56	0,40
2,34	2,33	0,00
2,37	1,91	0,29
2,15	1,93	0,34
2,32	1,16	0,24
2,13	1,33	0,79
2,26	1,70	0,39
2,17	1,80	0,00
2,17	1,28	0,46
2,21	1,45	0,68
2,12	1,76	0,00
2,29	2,11	0,24
2,21	1,50	0,26
2,25	1,42	0,70
2,20	1,02	0,91
2,30	1,10	0,88
2,20	1,49	0,00
2,27	1,61	0,32
2,38	2,34	0,00
2,11	1,18	0,57
2,16	1,01	0,61
2,11	1,45	0,00
2,33	1,05	0,78
2,28	1,11	0,52
2,12	1,43	0,44
2,37	1,59	0,30
2,17	1,29	0,57
2,10	1,69	0,22
2,34	1,56	0,28
2,28	1,58	0,39
2,22	1,27	0,61
2,18	1,48	0,38
2,17	1,37	0,50
2,25	1,46	0,54
2,27	1,73	0,45
2,13	1,94	0,38
2,32	2,23	0,00
2,37	1,30	0,58
2,21	1,43	0,54
2,13	1,38	0,38
2,18	2,01	0,00
2,14	1,39	0,42
2,19	1,44	0,55
2,22	1,42	0,42
2,35	2,33	0,00
2,36	2,35	0,00
2,13	1,71	0,29
2,18	1,68	0,41
2,20	1,41	0,72
2,13	1,47	0,00
2,38	1,50	0,57
2,29	1,57	0,55
2,24	1,34	0,00
2,24	1,43	0,51

2,13	1,21	0,55
2,15	2,25	0,00
2,16	1,66	0,24
2,18	2,05	0,00
2,32	0,75	0,62
2,14	2,18	0,00
2,10	1,40	0,33
2,17	1,68	0,50
2,14	1,99	0,00
2,33	1,76	0,66
2,28	1,64	0,51
2,29	2,09	0,00
2,23	1,47	0,26

Table 2. The measured subnuclear distances in µm of the PJ1185 strain grown in EMM-N. First row, diameter of the cell (d), second row, distance between the GFP and the SPB, third row, distance between the GFP and the NM.

	No. cells	Mean d	Median d	Mean GFP-SPB	Median GFP-NM
PJ1185 +N	69	2.239	2.230	1.777	0.000
PJ1185 -N	70	2.223	2.210	1.587	0.390

Table 3. Descriptive statistics of the observed subnuclear distances in strain PJ1185 before (+N) and after (-N) nitrogen starvation. First row: number of cell measured, second row: mean diameter (d), third row: median diameter (d), forth row: mean distance between the GFP and SPB, fifth row: median distances between the GFP and NM.

Discussion

During the last decade the use of live cell imaging to monitor cellular events has become increasingly popular. It started with the use of Green Fluorescence Protein from jellyfish *Aequorea victoria* and now many different fluorochromes are available emitting fluorescence through a wide spectra from cyan (475 nm) to far red (648 nm) [11]. One of the major advantages of live cell imaging over immunofluorescence is that the cells are not fixed by formaldehyde or ethanol/acetone treatment before microscopy, hence avoiding possible artefacts from the fixation process. In addition, live cell imaging offers the possibility to follow individual cells and take pictures at frequent intervals during hours or days of incubation, making it possible to get movies of cellular events [12]. Mammalian cells have the advantage of having a larger size, with diameters of around 100 µm, as compared to the smaller yeast cell with a diameter of about 3-4 µm. On the other hand, the advantage with yeast is the easily manipulated genome. Homologous recombination occurs very efficiently in yeast and is used to fuse proteins of interest to different fluorochromes [3]. Moreover, using targeted integration of *lacO* sequences and subsequent expression of LacR-GFP protein allows the detection of a specific part of the genome within the cell nucleus [2]. Using *S. pombe* cells in microscopy has additional advantages since they are natural unicellular organisms that grow with a fast generation time in low-cost cell culture conditions. Moreover, *S. pombe* is an excellent eukaryotic model organism since it has metazoan homologous genes.

One of the major limitations of this technique is autofluorescence in the yeast cell disturbing the detection of the true signal. This problem can be overcome by using minimal growth media with filter sterilised glucose instead of autoclaved. In addition the yeast cells should be grown for 2 days in log phase before mounting them. The protocol presented here offers a relatively simple, but yet quantitative method to determine the subcellular localisation of proteins within the yeast cell. Moreover by taking pictures at different time points we can follow cellular events.

Disclosures

We have nothing to disclose.

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