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Copy number analysis of the yeast histone deacetylase complex component Cti6 directly in living cells

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Abstract

Proteins are the one of the key components of cellular life that play a crucial role in most biological processes. Therefore, quantification of protein copy numbers is essential for revealing and better understanding of cellular behaviour and functions. Here we describe a single-molecule fluorescence-based method of protein copy numbers quantification directly in living cells. This enables quick and reliable estimations and comparison of protein of interest abundance without implementing large-scale studies.

Key words: histone deacetylase, single-molecule, copy number, living cells

1. Introduction

Proteins are one of the main functional components of cells. Protein abundance is the key in regulation of protein-protein and protein-nucleic acids interactions as well as enzymatic reactions which comprise most biological processes. This makes quantification of protein copy numbers essential for revealing and better understanding of cellular behaviour and functions.

Initially, methods for protein numbers estimation relied on gene expression measurements, such as microarray hybridisation (***1***). However, protein homeostasis resembles a balance between protein production, including mRNA processing and translation, and degradation. Therefore, reliable quantification can be obtained only by counting protein numbers directly. Multiple large-scale high-throughput studies on eukaryotic proteome, mainly that of the budding yeast *Saccharomyces cerevisiae*, have been performed using various methods. This

includes quantitative mass spectrometry, western blotting, fluorescence-based methods, such as wide field and confocal microscopy approaches as well as flow cytometry (***2***–***8***).

We report an easy manual method based on a single-molecule fluorescence data, which allows for a quick estimation of protein numbers in living eukaryotic cells. We have applied such an approach to the Cti6 protein of the chromatin remodelling system in the budding yeast *S. cerevisiae*. Cti6 is a component of the large histone deacetylase complex Rdp3L and participates in multiple cellular processes including iron tolerance and metabolism maintenance (***9***). Cti6 plays an important role in gene expression regulation via its binding to the SAGA activator complex (***10***). Moreover, Cti6 has been reported to convert one of the largest repression machineries, the Ssn6-Tup1 global co-repressor complex, into an activator (***9***, ***11***).

Here we report in detail all necessary steps to estimate molecular copy numbers of a protein of interest in living yeast *S. cerevisiae* cells based on the fluorescent signal. We describe endogenous genomically integrated labelling of the Cti6 protein with a monomeric form of GFP, mGFP, which contains the *A206K* point mutation (***12***). We used a super-resolution Slimfield microscopy set-up to visualise Cti6-mGFP directly in living cells. We then applied previously reported MATLAB code (***13***, ***14***) to track fluorescent complexes and estimate intensity of a single fluorescent molecule. We then show how these data can be used to estimate Cti6 copy numbers per cell.

2. Materials

2.1 Yeast strains

1. The BY4741 wild type yeast *S. cerevisiae* cells, pmGFPS plasmid (***14***) (*HIS3, GFPmut3 S65G, S72A, A206K*).

2. 40% (w/v) glucose solution.

3. Yeast Nitrogen Base (YNB) media (with complete amino acid supplement and -his): 1.7 g/l yeast nitrogen base without amino acids and $(NH_4)_2SO_4$, 5 g/l $(NH_4)_2SO_4$, complete amino acid supplement or -his amino acid supplement as indicated by manufacturer, pH 5.8-6.0, supplemented with 4% w/v glucose (added after autoclaving). To prepare YNB plates, add 20 g/l agar before autoclaving.

4. Yeast Peptone Dextrose (YPD) liquid medium (10 g/l yeast extract, 20 g/l bacto-peptone) supplemented with 4% w/v glucose (added after autoclaving). To prepare YPD plates, add 20 g/l agar before autoclaving.

5. Phusion high fidelity DNA polymerase 2U/μl kit, dNTP mix.

6. Primers: [Fig 1 near here] for mGFP::his3 fragment amplification, Forward (Fw) and Reverse (Rv) primers contain ~16-20 bp of the 5′ and 3′ ends, respectively, of the mGFP::his3 fragment from pmGFPS plasmid plus ~50bp sequences up- (Fv) and downstream (Rv) of the Cti6 STOP codon (Fig. 1a). For the conformation PCR, Forward (Fconf) and Reverse (Rconf) primers are

sequences of ~16-20 bp located at the 3′ end of the *CTI6* gene and 5′ end of the mGFP::his3 (Fig. 1b).

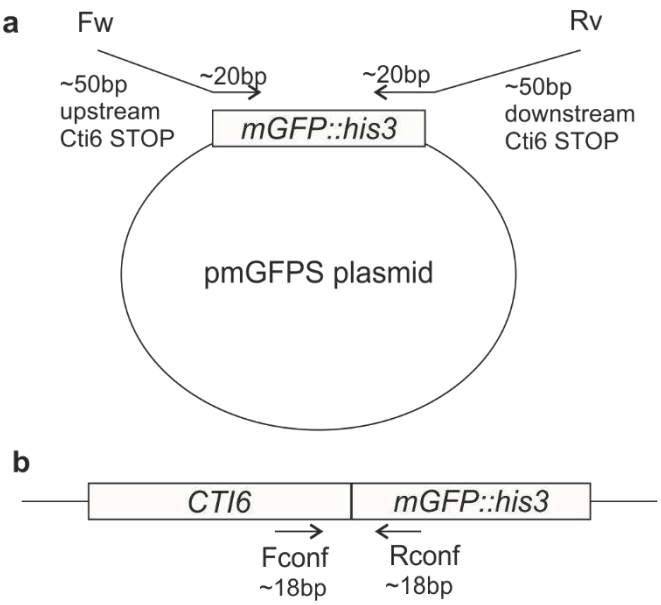

Fig. 1 *Primer design for (**a**) amplification of a mGFP::his3 fragment for further genomic integration to label the Cti6 protein and (**b**) verification of a correct insertion of the fluorophore sequence.*

7. 0.8% agarose gel with Midori Green or any other nucleic acid stain in 0.5x TAE buffer.

8. 1kb DNA ladder, 6x loading buffer.

9. 1M LiAc water solution.

10. 100mM LiAc water solution.

11. 50% w/v PEG-4000 solution in water.

12. 10 mg/ml single strand DNA (we use salmon sperm DNA).

13. 0.02% SDS solution in water.

14. Standard epifluorescence microscope. We used Zeiss Axio Observer.Z1 inverted microscope with Apotome and Axiocam 506 345 camera, and a Plan-Apochromat 100x/1.40 Oil DIC M27 objective.

2.2 Super-resolution microscopy

1. We utilised the super-resolution fluorescence Slimfield microscope, with ~6 W/cm^2 excitation intensity directed onto a sample, 50mW Obis 488 nm laser, 100x 1.49 TIRF 1.49 NA oil immersion objective, EMCCD camera (Prime 95B scientific CMOS; Teledyne Photometrics) with 50 nm/pixel magnification (***14***, ***15***).

2. 125 µl Gene Frames.

3. Plasma-cleaned BK7 glass microscope coverslip (22 × 50 mm).

4. 2x YNB complete liquid medium supplemented with 8% w/v glucose.

5. 2% agarose solution in Milli Q water.

2.3 For the computational analysis, we used a combination of MATLAB, ImageJ FiJi and Excel software.

3. Methods

3.1 Fluorescent labelling of the Cti6 protein

1. Streak the BY4741 wild type cells from a frozen stock, using a sterile pipette tip on a freshly prepared YPD plate, and incubate at 30 °C for at least 24 h.

2. Set a culture in a 14 ml tube by inoculating 3 ml of YPD with cells grown a YPD plate. Incubate at 30 °C, 180 rpm, overnight.

3. Amplify the mGFP::his3 fragment from the pmGFPS plasmid (***14***) by using Fw and Rv primers, dNTP mix and Phusion high fidelity DNA polymerase 2U/μl kit as recommended by manufacturer. With following PCR programme:

Initiation	98.0°C	– 30 sec	
Denaturation	98.0°C	– 10 sec	30 cycles
Annealing	60.6°C	– 20 sec	
Elongation	72.0°C	– 60 sec	
Final elongation	72.0°C	– 5 min	
Keep at 4°C			

Verify the size of the fragment (here ~1920 bp) on 0.8% agarose gel by mixing 5 μl of a PCR reaction mix with 1 μl of 6x Loading Dye and applying onto a gel (100V, 25 min in 0.5x TAE buffer). Use 1kb DNA ladder as a reference for the size of the amplified fragment. Store at -20°C until further use.

4. Inoculate 50ml of YPD medium with the overnight culture to the final OD_{600} ~0.2. Grow until mid-logarithmic phase, OD_{600} ~0.7-1.0.

5. Transform the culture with 70 μl of the PCR mix containing mGFP::his3 fragment by standard LiAc protocol (***16***). In brief, mix 50 μl of washed with water and then 100 mM LiAc cells with 240 μl of 50% w/v PEG-4000, 36 μl of 1M LiAc, 5 μl of 10 mg/ml salmon sperm DNA and 70 μl of the PCR mix

containing mGFP::his3 fragment. Incubate the entire mix at 30°C for 30 min, then heat-shock at 42°C for 25 min. Wash (***Note 1***) and plate the transformation mix onto YNB-his plates. Incubate at 30°C for 3 days.

6. Confirm the colonies for the green signal under the microscope. We used Zeiss Axio Observer.Z1 inverted microscope with Apotome and Axiocam 506 345 camera, and a Plan-Apochromat 100x/1.40 Oil DIC M27 objective.

7. Verify correct integration of the mGFP::his3 fragment by confirmation PCR (in detail below).

8. Streak correct clones on a new YNB-his plate (***Note 2***), incubate for 24 h at 30°C. Store at 4°C. For longer storing, collect grown cells, mix in a cryo tube with 1ml of YP+20% glycerol solution, put into -80°C freezer.

3.2 Confirmation PCR

1. Extract genomic DNA: take a bit of a transformant into 20 μl of 0.02% SDS, heat at 98°C for 3 min. Spin down at 6000 rpm, 10 sec. Mix 10 μl of the supernatant with 10 μl of MiliQ water.

2. Perform PCR using Phusion high fidelity DNA polymerase 2U/μl kit as recommended by manufacturer. With following the PCR programme as above but using 58.9°C as an annealing temperature. Verify the size of the

fragment (here ~175 bp) on 0.8% agarose gel (100V, 25 min in 0.5x TAE buffer).

3.3 Preparing cells for microscopy

1. Set overnight cultures of BY4741 wild type and Cti6-mGFP strains in two 14 ml tubes by inoculating 3 ml of YNB complete with cells grown on plates. Incubate at 30 °C, 180 rpm.
2. In the morning, subculture the cells into 2x 3ml of fresh YNB complete medium to the final OD_{600} ~0.2. Grow for four hours at 30 °C, 180 rpm.
3. Spin cells down at 3000 rpm, 1 min, remove the supernatant. Resuspend cells in 0.5 ml of fresh YNB complete medium.

3.4 Preparing microscopy slides using 125 µl Gene Frame

1. Remove the larger plastic cover and apply the frame onto a standard microscopy glass slide.
2. Prepare 0.5 ml of 2x YNB complete medium supplemented with 8% w/v glucose in a 1.5 ml Eppendorf tube.

3. Add 0.5 ml of melted 2% agarose water solution, quickly mix by pipetting up and down, take 0.5 ml of obtained mixture and add onto the slide within the Gene Frame.

4. Quickly apply a plastic cover slip from the Gene Frame package to remove the access and evenly distribute the solution within the well. Let sit for a few min.

5. Carefully remove the plastic cover slip by sliding it off.

6. Remove the smaller plastic cover from the frame.

7. Add ca 5 µl of the prepared cell in droplets across the entire pad. Leave to dry for 3-5 min.

8. Cover the pad with a plasma-cleaned BK7 glass microscope coverslip (22 × 50 mm), avoid air bubbles.

3.5 Data acquisition

1. Place the sample under the Slimfield microscope and locate a cell, adjust the focus to the middle of the cell.

2. Acquire 2 brightfield images with 10ms exposure time.

3. Turn off the brightfield and acquire 1000 images at 5 ms exposure time with the 488nm laser at 20mW power to obtain images of Cti6-mGFP.

4. Repeat for at least 30 cells.

5. Repeat the whole procedure for the non-fluorescent parental strain BY4741, this will be used for autofluorescence correction during the copy number estimation.

3.6 Copy number analysis

1. Load the TIF file containing fluorescence data acquisition into MATLAB as *n x m* pixels by *p* frames and perform tracking of individual fluorescent spots as described previously (***17***).

2. We estimate the fluorescence intensity of an individual mGFP molecule (*Isingle*) based on the intensity distribution of the Cti6-mGFP spots identified in the last 2/3 of the acquisition frames (***Note 3***). [Fig 2 near here] As at this point, single photobleaching events become more apparent and detectable,

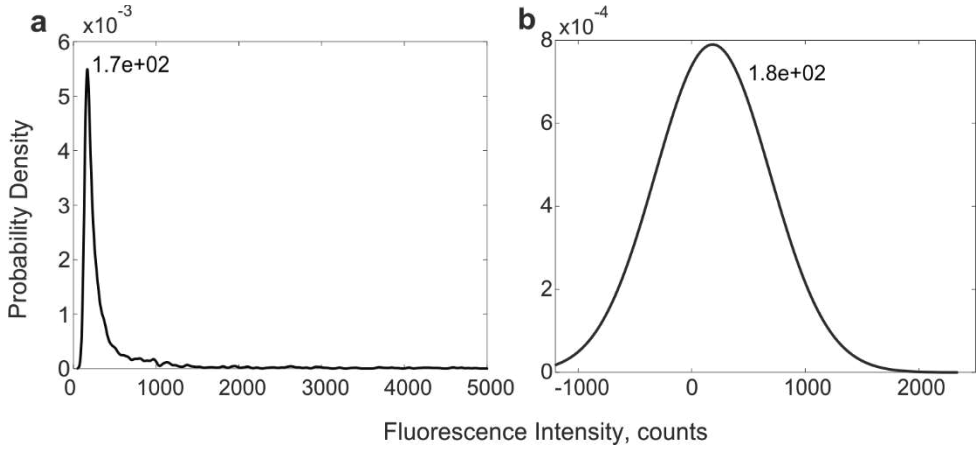

Fig. 2*. Distribution of all Cti6-mGFP spot intensities (**a**) and spots identified towards the end of photobleaching (**b**). Peak values are indicated.*

therefore, the most common value (the peak) represents the intensity of a single fluorophore (Fig. 2).

3. Load TIF file containing fluorescence data acquisition into ImageJ Fiji software and open the brightest frame.

4. Choose the "oval" selection shape on the ImageJ tool bar and draw a circle around the cell. Keep the same size of the selection for all measurements.

5. To obtain numeric values of total fluorescence intensity of the cell by pressing 'Ctrl' + 'M' (Analyse → Measure). Record Raw Integrated Density number (RawIntDen) in Excel. Repeat for all files containing fluorescence data of BY4741 Cti6-mGFP cells.

6. Repeat steps 1-5 for the BY4741 wild type fluorescence files. Calculate the average autofluorescence.

7. Subtract the average autofluorescence value from each RawIntDen value recorded from the BY4741 Cti6-mGFP cells.

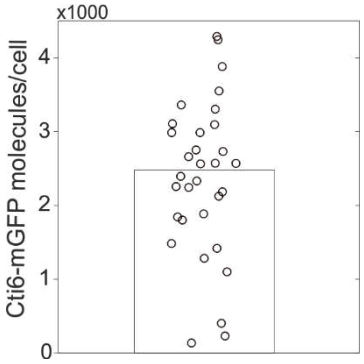

Fig. 3*. Jitter plot of Cti6-mGFP copy numbers per cell across a population of 32 individual cells. Bar indicates the median.*

8. To estimate the copy numbers of Cti6-mGFP per cell, divide obtained values by *Isingle*.

Figure 3 [Fig 3 near here] shows distribution of Cti6 numbers per cell across a population of 32 cells. We estimated the mean number of Cti6 molecules as 2368±1029.

4. Notes

1. To increase the transformation efficiency, prior plating, the cells can be washed and incubated in YPD medium supplemented with 4% w/v glucose for 1 h at 30°C, 180 rpm.
2. As the fragment is genomically integrated, it is very unlikely that it will be lost during cellular division. Therefore, once the correct clones were confirmed, it is not mandatory to have a selective medium.
3. *Isingle* can also be estimated by imaging purified mGFP immobilised on a cover slip.

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