Published in final edited form as:

Methods Cell Biol. 2008 ; 85: 127–151. doi:10.1016/S0091-679X(08)85007-8.

Counting Kinetochore Protein Numbers in Budding Yeast Using Genetically Encoded Fluorescent Proteins

Ajit P. Joglekar, E. D. Salmon, and Kerry S. Bloom

Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599

Abstract

Genetically encoded fluorescent proteins are an essential tool in cell biology, widely used for investigating cellular processes with molecular specificity. Direct uses of fluorescent proteins include studies of the *in vivo* cellular localization and dynamics of a protein, as well as measurement of its *in vivo* concentration. In this chapter, we focus on the use of genetically encoded fluorescent protein as an accurate reporter of *in vivo* protein numbers. Using the challenge of counting the number of copies of kinetochore proteins in budding yeast as a case study, we discuss the basic considerations in developing a technique for the accurate evaluation of intracellular fluorescence signal. This discussion includes criteria for the selection of a fluorescent protein with optimal characteristics, selection of microscope and image acquisition system components, the design of a fluorescence signal quantification technique, and possible sources of measurement errors. We also include a brief survey of available calibration standards for converting the fluorescence measurements into a number of molecules, since the availability of such a standard usually determines the design of the signal measurement technique as well as the accuracy of final measurements. Finally, we show that, as in the case of budding yeast kinetochore proteins, the *in vivo* intracellular protein numbers determined from fluorescence measurements can also be employed to elucidate structural details of cellular structures.

I. Introduction

Fluorescence microscopy is finding increasing usage in studies of diverse aspects of cell biology at the cellular as well as the molecular level, with a variety of fluorescent probes available for studying molecular function (Giepmans *et al.*, 2006). Genetically encoded fluorescent proteins have become the most widely used fluorophores by cell biologists (Chudakov *et al.*, 2005). The green fluorescent protein (GFP) was the first to be optimized as a genetically encoded fluorescent marker for use *in vivo* (Tsien, 1998). The exploding number of available fluorescent proteins with characteristics tailor-made to suit experimental needs (Miyawaki, 2004; Miyawaki *et al.*, 2005; Sawano and Miyawaki, 2000; Zhang *et al.*, 2002) has allowed researchers to devise elegant ways of employing them to reveal cellular processes. Their typical uses range from *in vivo* protein localization and dynamics (Lippincott-Schwartz and Patterson, 2003), characterizing intracellular chemistry (Kohl and Schwill, 2005; Lippincott-Schwartz *et al.*, 2001; Schwill, 2001), to studying gene expression and regulation patterns (Bar-Even *et al.*, 2006; Colman-Lerner *et al.*, 2005; Raser and O'Shea, 2005; Rosenfeld *et al.*, 2005). Besides their utility in studying spatiotemporal protein localization patterns within a cell, quantification of intracellular protein concentration is intuitively the simplest application of genetically encoded fluorescent proteins in cell biology.

Estimation of intracellular concentration of fluorophores is commonly employed using flow cytometry (Huh *et al.*, 2003; Newman *et al.*, 2006). This technique measures whole-cell fluorescence signal to deduce the concentration of a protein or DNA within the cell. Flow cytometry works well for bright signals in samples that have relatively low autofluorescence

and has the advantage of rapidly accumulating very large datasets. However, it may not provide the desired flexibility necessary for the accurate measurement of proteins that have low cellular abundance, with protein numbers per cell ranging from a few to a few hundred protein molecules (Newman *et al.*, 2006). This is commonly the case for studies involving prokaryotes and lower eukaryotes such as budding and fission yeast. In recent years, a number of microscopic imaging-based methods have been devised for this purpose. These methods typically involve high numerical aperture (NA) objectives for imaging, and sensitive, low-noise cooled CCD cameras for image acquisition. Current technology allows the observation of protein expression one molecule at a time *in vivo* (Xie *et al.*, 2006; Yu *et al.*, 2006).

There are two important considerations that must be addressed while developing a quantitative fluorescence microscopy method for counting protein numbers. The first involves the development of an accurate technique for the quantification of the fluorescence signal that is based on features of the biological structure being studied, and the characteristics of the imaging system. The second factor is to obtain a calibration standard that allows for the accurate conversion of measured fluorescence signal into the corresponding number of fluorophores. While the details of the first factor are largely dictated by the imaging optics, diverse methodologies have been developed over the years to address the need of a good calibration standard. The choice of a particular calibration standard can play a significant role in determining the accuracy of the protein counts obtained. A fluorescence signal measurement and conversion method designed with these considerations can yield valuable data on intracellular protein numbers.

In this chapter, we describe a technique for accurately counting the numbers of kinetochore proteins in budding yeast by measuring the fluorescence signal from fluorescently tagged kinetochore proteins. First, the criteria for choosing optimal fluorescent proteins for counting intracellular protein numbers are discussed briefly. We then describe the sample preparation method and the microscope setup used. The next section discusses characterization of the microscope performance, which is critical in developing an appropriate signal measurement scheme. Fluorescence signal quantification method for the budding yeast kinetochore cluster, along with the obtained results, is then described. The last section contains a discussion of a more generalized fluorescence signal measurement method that may be used for proteins that are diffusely distributed within a cell, as well as the sources of errors in fluorescence signal measurements. This section also includes a survey of the varied fluorescence signal calibration standards that have been developed to convert the fluorescence signal into the corresponding number of proteins.

II. Counting Kinetochore Protein Numbers in Budding Yeast

The eukaryotic kinetochore is a highly complex protein structure composed of more than 60 different proteins (McAinsh *et al.*, 2003; Meraldi *et al.*, 2006). It establishes attachment of microtubule plus-ends with the centromeric DNA during mitosis, and generates force necessary to move and segregate chromosomes. The end-on attachment of a microtubule plus-end with centromeric DNA requires at least eight different proteins and protein complexes. To understand how these proteins assemble together to make a functional kinetochore-microtubule attachment, it is necessary to understand their arrangement within the microtubule attachment site at the kinetochore. Although electron microscopy has revealed the overall structure of the kinetochore, the arrangement of the protein complexes within this structure remains unknown. The number of copies of each protein or protein complex involved in making a functional kinetochore-microtubule attachment is critical for understanding the molecular architecture of the kinetochore. The budding yeast (*Saccharomyces cerevisiae*) as a model organism provides some unique advantages as an experimental system for counting kinetochore protein numbers *in vivo* using quantitative fluorescence microscopy.

A wealth of information about the composition of the budding yeast kinetochore is now available. The budding yeast kinetochore is a relatively simple structure with only one microtubule attachment site as compared with vertebrate kinetochores that have multiple microtubule attachments. Each kinetochore is based on ~300 base pair long DNA sequence wrapped around one centromeric nucleosome containing centromere-specific histone Cse4p (human homolog CENP-A). In metaphase, the centromeric DNA is stably attached to the plus-end of one microtubule by nine other linker proteins or protein complexes (Fig. 1). Since each yeast kinetochore supports only one stable microtubule attachment in metaphase, the copy number of each protein complex per kinetochore can be directly useful in understanding the molecular architecture of the microtubule attachment site. The stability of microtubule attachment raises the possibility that the protein complexes that make up the microtubule attachment site may also be stably associated with the kinetochore. Most of the proteins in this linkage are conserved in all eukaryotes including humans (McAinsh *et al.*, 2003; Meraldi *et al.*, 2006). Therefore, protein architecture within a kinetochore-microtubule attachment can also be expected to be conserved from budding yeast to humans.

The versatile molecular biology and genetics of budding yeast provides a critical advantage for protein number evaluation through the measurement of fluorescence signal from fluorescently tagged proteins. Most proteins can be easily tagged at the C-terminus with a fluorescent protein by insertion of the gene sequence at the endogenous locus. Thus, the fusion protein is the only species of protein produced within the cell. The protein level can be expected to be similar to the native strain as the fusion protein expression is controlled by the native promoter. Furthermore, the fusion protein can be considered as functionally equivalent, since it replaces the native protein without an observable effect on chromosome segregation or gross cell growth. Therefore, the protein counts obtained with fluorescence microscopy can be expected to accurately reflect the functional needs in the cell. This critical advantage is absent in vertebrate systems, since expression of a fusion proteins from the native promoter has proven difficult. Therefore, the fluorescently tagged protein is typically expressed with the help of an extra copy of the gene fused to the gene for a fluorescent protein from an artificial promoter. This results in nonnative protein expression of the fluorescently tagged protein along with the native untagged protein. Therefore, suitable control experiments must be designed to account for the presence of two species of proteins within the cells, and possibly preferential recruitment of these proteins at the site of action. The use of budding yeast as a model system avoids these complications.

The geometry of the budding yeast spindle in metaphase and anaphase/telophase facilitates accurate evaluation of the fluorescence signal from fluorescently labeled kinetochore proteins (Fig. 2A and B). The metaphase spindle is ~1500 nm in length, with two clusters of sister kinetochores separated by 800 nm and positioned on either side of the spindle equator (Fig. 2C). Each cluster consists of 16 kinetochores that are distributed over a 200–300 nm region. Each kinetochore is stably connected to the plus-end of one microtubule that is anchored at its minus end within a spindle pole body. In anaphase, the kinetochore microtubules shrink to very short lengths (~50 nm), pulling their kinetochores very close to a spindle pole body. The clusters of sister kinetochores become highly separated by spindle elongation to a length of 8–10 μ m.

Budding yeast also provides an excellent calibration standard, which is critical for converting the fluorescence signal into the number of fluorophores accurately. As mentioned earlier, the yeast kinetochore is built around a single centromeric nucleosome that contains two Cse4p molecules (Collins *et al.*, 2004; Joglekar *et al.*, 2006; Meluh *et al.*, 1998). Thus, the signal from a kinetochore cluster expressing Cse4p-GFP represents the fluorescence of 32 GFP molecules (16 kinetochores with 2 Cse4p-GFP molecules per kinetochore). The spatial protein distribution within the cluster for all the kinetochore proteins is nearly identical to that of Cse4p,

which avoids some of the potential complications in fluorescence signal measurement. We have developed a ratiometric method for counting the number of GFP-tagged kinetochore proteins. This method evaluates the fluorescence signal for a GFP-tagged kinetochore protein, and compares it with the signal for Cse4p-GFP by obtaining a ratio of the two fluorescence signals. The conversion of this ratio into protein number is then a straightforward task, since the exact number of Cse4p per kinetochore is known.

Figure 3 compares the fluorescence intensity from kinetochore clusters in metaphase and anaphase/telophase cells expressing the different GFP-tagged kinetochore proteins (shown in green in Fig. 1). The images in both the panels were obtained with nearly identical imaging conditions. The intensity from the representative protein can be expected to accurately reflect the number of copies of the complex with which it is associated (Meraldi *et al.*, 2006). Even a cursory examination of the images clearly shows that different proteins are incorporated within the kinetochore in different numbers. An image analysis method is necessary to accurately evaluate the fluorescence signals, and convert them into absolute numbers of corresponding proteins.

A. Optimal Fluorescent Proteins

Selection of the appropriate fluorescent protein is critical for its use as a faithful reporter of intracellular protein concentration (Shaner *et al.*, 2005). The following characteristics of the fluorescent protein are highly desirable: (1) a high and constant efficiency at the typical physiological temperature, (2) a maturation time faster than the temporal dynamics of the protein under investigation, (3) a high molecular brightness to produce a robust signal that is also insensitive to its microenvironment, and (4) a low bleaching rate. EGFP was the first fluorescent protein to be optimized as a suitable genetically encoded fluorescent protein for quantitative microscopy (Patterson *et al.*, 1997; Piston *et al.*, 1999). It is an ideal reporter protein in budding yeast because of its fast maturation time, high folding efficiency, and satisfactory molecular brightness. Its color variants YFP and CFP provide suitable alternatives, although properties such as bleaching rates are not as good as the properties of EGFP. YFP can be useful because of a higher molecular brightness, and because it allows the use of filters that enable a greater rejection of autofluorescence background in certain cases (Wu and Pollard, 2005). Low bleaching rate is especially important when counting low protein numbers, and when temporal changes in protein numbers are being studied. Bleaching also becomes an issue if multiple image slices are necessary to cover the entire thickness of a cell. Table I lists relevant properties of fluorescent proteins that are particularly suited for counting experiments.

III. Sample Preparation

The budding yeast strains used in our study were created by tagging the endogenous copy of the kinetochore protein gene with EGFP at the C-terminus (Longtine *et al.*, 1998). A strain made in this manner shows wild-type growth characteristics at 25 °C in complete media (YPD), indicating that the EGFP-tagged kinetochore protein can functionally substitute the native, untagged protein. Standard methodologies have now been established for *in vivo* imaging of fluorescent proteins in budding yeast (Bloom *et al.*, 1999). In brief, the cells are grown to mid-log phase in YPD at 25 °C. For microscopy, the cells are spun down, washed in water, and then resuspended in filter-sterile synthetic media (SD). Coverslips are immersed in 1 M NaOH overnight. After washing them thoroughly with distilled water, a thin layer of 0.5 mg/ml concanavalin A (cat # C7275, Sigma) solution (10 mM phosphate buffer, 1 mM CaCl₂, pH 6.0) is then applied on the coverslip for ~20 min. The coverslips are washed again with distilled water and allowed to dry. In each experiment, cells belonging to two strains—one expressing the protein of interest tagged with GFP and the other expressing Cse4p-GFP—are mixed together in approximately equal concentration. Five microliters of this mixture is then spread

over a concanavalin A coated coverslip. The coverslip edges are then sealed with VALAP (a mixture of equal amounts of vaseline, lanoline, and paraffin) to prevent media evaporation.

Sample preparation protocols used in live cell microscopy strive to maintain optimal conditions for cells under the microscope. For optimal fluorescence microscopy however, a low but uniform background fluorescence due to growth media is also desirable. Immobilizing yeast cells with concanavalin A avoids the use of thin gelatin slabs that are commonly used to hold the yeast cells in place during live-cell microscopy (Bloom *et al.*, 1999). We found that the autofluorescence from gelatin slabs increases with depth, which makes accurate evaluation of fluorescence signals from kinetochore clusters difficult.

IV. Microscope and Image Acquisition System

We used wide-field epifluorescence microscopy and digital imaging for counting kinetochore proteins in budding yeast. The common considerations in selecting the critical components for a wide-field epifluorescence microscope have been previously described (Salmon *et al.*, 2003). For fluorescence signal measurements in budding yeast, we used a Nikon TE 2000-U microscope (Nikon, Melville, NY) equipped with a 1.4 NA, 100× DIC oil immersion objective. We used the HQ filter cube for EGFP from Chroma (Chroma, Rockingham, VT; exciter: HQ470/40x, dichroic: Q495LP, and emission: HQ525/50m). The microscope is equipped with an XY translation stage (LEP, Hawthorne, NY). The objective can be translated along the optical axis with a servo stepper motor (LEP). Images were acquired with an Orca ER cooled CCD camera (pixel size of 6.47 μm , Hamamatsu). The camera was operated in the 2×2 binning mode. Only a 300×300 pixel region at the center of the field of view was acquired. For each selected field, 21 Z sections were taken, with a distance of 200 nm separating successive image planes. Image acquisition was carried out with Metamorph (Molecular Devices, Downington, PA). Image analysis was done by using a custom written graphical user interface in MatLAB (MatLAB, Natick, MD).

The selection of appropriate imaging conditions is carried out to maximize the signal-to-noise ratio of the images. It depends on the characteristic of the fluorescent protein as well as the imaging system. The response of the fluorescent protein to the excitation intensity is the first important characteristic. GFP signal increases nonlinearly with the excitation intensity, and saturates beyond a critical intensity, behavior that is typical of three-state systems (Kues *et al.*, 2001). This nonlinear response becomes especially important when comparing fluorescent protein signals acquired at two different intensities. Another characteristic of a typical fluorophore is fluorescence or concentration quenching. Fluorescence quenching is the effect of nonradiative depletion of fluorophores in the excited state that becomes pronounced at high fluorophore concentrations. Fluorescence quenching thus distorts the expected linear relationship between fluorescent protein concentration and fluorescent protein signal. As discussed later, we found that local EGFP concentrations (over the range considered in our study) do not distort the fluorescence signal. The excitation intensity and exposure time were optimized to minimize photobleaching, and to allow for optimal usage of the dynamic range of the camera. An exposure of 400 ms with the Orca ER camera allowed us to image kinetochore clusters with the lowest signal with a satisfactory signal-to-noise ratio, while allowing the recording of fluorescence signal of the most abundant proteins without pixel saturation. This exposure was used for all the measurements. For a large field of view, it is important to evaluate the variation in the excitation intensity as a function of position in the object plane in wide-field microscopy. These variations can be characterized by imaging a uniformly labeled surface and applying a “shading correction” to the acquired images. Depending upon the amount of variation over the necessary imaging area and the desired accuracy, advanced image calibration methods may be used (Ghauharali and Brakenhoff, 2000; Zwier *et al.*, 2004). In our experiments, we acquired images only from a square in the center of the image plane

representing less than 10% of the total area of the image plane. The excitation intensity can be assumed to be spatially invariant over this small region.

The choice of a digital CCD camera plays an important role in deciding both the accuracy and sensitivity of fluorescence signal measurements. The two critical characteristics of a CCD camera to consider are: (1) the photon conversion efficiency of the CCD chip and (2) camera noise. Modern electronics makes electronic noise less significant in comparison with the background noise from the sample or microscope optics. The linearity of CCD response to the incident intensity is excellent, thus making its use in signal quantification straightforward.

An optimal balance between the spatial resolution in an image and the signal-to-noise ratio of that image is important for carrying out fluorescence signal measurements. The process of recording a microscope image with a CCD pixel array results in a convolution of the image by a top-hat function corresponding to the dimensions of each CCD pixel. The pixel size of the CCD camera used thus is an important parameter in determining the magnification needed in the image plane (Piston, 1998; Salmon *et al.*, 2003). With 100x magnification of the objective and a pixel size of 6.47 μm , each pixel corresponds to 65 nm in the object plane. Objective magnification and camera pixel size thus together decide the sampling frequency for the objective. The sampling frequency can be varied within limits by either changing the objective magnification or focal length of the tube lens or by electronic binning of neighboring pixels on the camera. The sampling frequency is important especially when measuring the fluorescence signal from subdiffraction objects. For imaging kinetochore clusters in budding yeast, we found that 2×2 binning of the camera chip provided sufficient spatial resolution to clearly distinguish metaphase clusters, while maintaining a sufficiently high signal-to-noise ratio for accurate signal measurement at moderate excitation intensity.

V. Measurement of Fluorescence Signal

A. Characterization of the Point Spread Function of the Objective

An objective produces a diffraction pattern in the form of an Airy disk in the image plane of a point source of light from the specimen such as a single fluorophore (Agard *et al.*, 1989; Inoue and Springer, 1997; Taylor and Salmon, 1989). The intensity distribution in the XY plane at focus along the Z axis is given by:

$$I = \left(\frac{J_1(R \times r)}{R \times r} \right)^2$$

$$R = \frac{0.61\lambda}{NA}, \quad r = \sqrt{x^2 + y^2} \quad (1)$$

where J_1 is the Bessel function of the first kind, λ is the wavelength, and NA is the numerical aperture.

The dimensions of this diffraction pattern, known as the Point Spread Function (PSF), are determined by the NA of the objective and the wavelength of fluorescent light. In the image plane, the Airy disk intensity distribution can be closely approximated by a two-dimensional (2-D) Gaussian function given by:

$$I = A e^{-(x^2/2\sigma_x^2)} e^{-(y^2/2\sigma_y^2)} \quad (2)$$

where σ_x , σ_y are the standard deviation in X and Y direction.

The spread of the Gaussian function is given by its standard deviation (σ_x , σ_y). The total signal from a point source (or from a subdiffraction object) is thus spread over the area of the PSF,

and thus can be evaluated by integrating the intensity over this entire region. The $\pm 2\sigma$ limits include $\sim 96\%$ of the area under a 1-D Gaussian curve ($\sim 90\%$ in 2-D), and is commonly used as the characteristic dimension for the distribution. These limits correspond to a circle with 444 nm diameter for an image of a point source of light emitting at 510 nm and recorded with a 1.4 NA objective. It should be noted that for high NA oil immersion objectives, the PSF approximates the theoretical PSF only very close to the coverslip surface. Spherical aberrations due to the mismatch in the refractive indices of glass and aqueous media rapidly distort the PSF away from the coverslip surface (Gibson and Lanni, 1992) and reduces the overall intensity (see the description later) (Fig. 4A – C).

It is important to accurately characterize the PSF for the objective to be used in the image plane (XY) and along the optical axis (Z) to verify the expected performance from the objective. This was done by imaging fluorescent beads of subdiffraction size (100 nm Transfluobeads, Invitrogen) immobilized on the coverslip. It is important to open up the field diaphragm for these measurements. In epifluorescence microscopy, the same objective is used to excite the fluorophores and to collect the emission light. Therefore, the focus change required to image out-of-focus light will also change the illumination intensity. This effect depends inversely on the size of the field diaphragm (Hiraoka *et al.*, 1990) and can be minimized by opening up the field diaphragm for PSF characterization. A line-scan through the experimentally obtained PSF in the XY plane of focus can be fitted with a 1-D Gaussian function to obtain the standard deviation (σ) for the Gaussian function as the fit parameter. The PSF along the optical axis can also be similarly approximated with a Gaussian curve.

B. Characterization of Fluorescence Intensity Distribution for a Kinetochore Cluster and Signal Measurement

The next step in developing the fluorescence measurement technique for a kinetochore cluster is to accurately determine its fluorescence intensity distribution in the image plane (the in-focus XY plane) and along the optical axis of the microscope (Z axis). This was done by recording the intensity distribution along a line through the maximum intensity pixel in an in-focus image of kinetochore clusters in cells expressing Nuf2p-GFP. The intensity distribution along the line was then fitted with a Gaussian function to obtain the standard deviation for the Gaussian as the fitting parameter. The standard deviation ($\sigma = 155$ nm) obtained from fitting the curve was used to determine the number of camera pixels to be included in the calculation of the integrated intensity from a kinetochore cluster (4×155 nm $< 5 \times 133$ nm, where 133 nm is the effective pixel size due to 2×2 binning). For metaphase kinetochore clusters, intensity had to be integrated over a larger area to account for the larger space occupied by the kinetochore clusters.

For accurate comparison, we obtained the average fluorescence signal for cells expressing Cse4p-GFP along with the average signal for the protein of interest from the same coverslip. Signal for a kinetochore cluster was defined as the integrated intensity for the cluster in the in-focus image plane. The in-focus image plane has the highest intensity, and thus provides the highest signal-to-noise ratio for intensity measurements. This approach also avoids the need for integrating the signal along the Z axis. An important step in signal calculation is the evaluation of the background signal. For an isolated fluorescent spot, accurate background evaluation can be carried out by using the method described by Hoffman *et al.* (2001). In brief, a box of the appropriate dimension is drawn concentrically around the box used for signal measurement. The dimension of this box is selected so as to equalize the area of the background and signal region. The integrated pixel intensity within the signal region after background subtraction was defined as the fluorescence signal from the kinetochore cluster. This method avoids errors due to inhomogeneities in the intracellular background levels. In cases where this

method cannot be adopted, for example, budding yeast cells in metaphase, the background region may be manually chosen to obtain an estimate of the true background.

The kinetochore clusters are situated at unknown distances from the coverslip surface in a yeast cell. Thus, a stack of optical sections through each cell along the optical axis must be acquired to capture an in-focus image of the kinetochore clusters within the cell. The precision with which the *XY* plane at the center/peak of the PSF along the optical axis can be captured depends on the separation between successive images along the *Z* axis (Fig. 3B, right graph). For example, the worst case situation with a 200-nm step size would be images captured 100 nm apart on either side of the actual peak in the PSF. This corresponds to a worst-case error of 8% of the maximum intensity. On average, random sampling of the PSF along the *Z* axis will result in an average underestimation of the measured signal by 4% of the maximum intensity. A 200-nm step size thus allows imaging through the entire thickness of the cell with minimal photobleaching during acquisition at the cost of a reasonably small measurement error. Depending on the signal-to-noise ratio and photobleaching rate for the fluorophore used, a smaller step size may be used.

VI. Validation of Measurement Method

To calibrate the linear response of the system, we took advantage of the known stoichiometry for constituent proteins within the NDC80 complex. Biochemical analysis of purified NDC80 complex from budding yeast shows that the four proteins within this complex have a 1:1:1:1 stoichiometry (Wei *et al.*, 2005). We constructed three yeast strains expressing either Nuf2p-GFP or Ndc80-GFP or both Nuf2p-GFP and Ndc80p-GFP. Using the method described earlier, we compared the fluorescence signal from kinetochore clusters in each case with the signal from a strain expressing Cse4p-GFP (Fig. 5A). As expected, the ratio of the average signal from Nuf2p-GFP was found to be identical to that for Ndc80p-GFP. The ratio for the strain expressing both Nuf2p-GFP and Ndc80p-GFP on the other hand was exactly twice in magnitude, thus validating our method.

These measurements also demonstrate that at least at these concentrations, there is no detectable quenching of the fluorescence signal. The GFP molecules are tethered by the kinetochore proteins in a stable protein structure. This insolubility may make the nonradiative transfer of energy from one fluorophore to another unlikely.

VII. Results

Table II lists the ratios for representative kinetochore proteins obtained by using the method described earlier. As mentioned earlier, we obtained the average fluorescence intensity for a kinetochore cluster for the reference (Cse4p-GFP) strain and the strain with the GFP-tagged protein of interest. Three experiments were performed for each protein in both metaphase and anaphase/telophase to obtain three ratios. The number of observations in each experiment for each strain was more than 20. We found that the coefficient of variation (standard deviation/mean) was sufficiently small (<0.25) for each data set. The values reported are the average of the ratios from the three experiments for each protein.

It must be demonstrated that the cumulative signal from a kinetochore cluster can be divided by 16 (the number of kinetochores in a cluster) to obtain the number of proteins per kinetochore. We verified this assumption by making use of a conditional dicentric chromosome. Inserted into chromosome III is an additional copy of the centromeric DNA sequence that is placed under the control of a GAL1 promoter. When the cells are grown on media with galactose as the carbon source, the transcription activity at the GAL1 promoter silences the additional centromere. In cells grown on glucose-containing media however, this additional centromere

becomes active, and builds a functional kinetochore on the same chromosome. Two active kinetochores on the same chromosome prevent the sister chromatids from segregating to their respective poles in anaphase in a fraction of cells. The lagging kinetochores, at positions away from the monocentric kinetochore clusters near the spindle poles, can be clearly imaged in cells expressing high copy number kinetochore protein such as Nuf2p-GFP (Fig. 5B). A comparison of the fluorescence signal from these single kinetochores with the cluster of 15 monocentric chromosomes close to the spindle pole bodies yielded an average ratio of 16 ± 2 , as expected. This experiment validates the assumption that each kinetochore contributes equally to the cumulative fluorescence signal from a cluster of 16 kinetochores.

The observed standard deviation of the signal about the mean value from metaphase or telophase kinetochore clusters contains important information about the variation in protein numbers from cell to cell. The observed standard deviation may also contain contributions from experimental errors in addition to the biological variation. We therefore analyzed the data to look for potential experimental sources of error to find that the distance of the kinetochore cluster away from the coverslip strongly affects the signal magnitude due to spherical aberrations that increase with depth as discussed above. As shown in Fig. 6, this effect does not depend on the absolute magnitude of the signal, and thus does not distort the ratio of two fluorescence signals. However, the resultant variation of the signal about the mean signal masks information about the variation in the protein number. To avoid this experimental source of error, we compared the fluorescence signals for kinetochore cluster pairs that had a relative separation along the optical axis of 600 nm or less. Table III lists the mean and standard deviation for three different strains spanning the range of signals measured in this study. As can be seen from the table, the difference in measured intensity values for these kinetochore clusters is small as compared with the total signal. The standard error of the mean fluorescence value based on this difference is also very small. It can be stated in terms of the number of GFP molecules, by using the average Cse4p-GFP signal (1945 counts for 32 GFP molecules at 16 kinetochores $\rightarrow$ 60 counts per GFP molecule). Thus, the difference between two kinetochore clusters in the same cell for Cse4p is ~ 4 GFP molecules out of 32, while that for Ndc80p-GFP + Nuf2p-GFP is 20 GFP molecules out of 256. This translates into a variation of less than one molecule per kinetochore for each protein. It should also be noted that the standard deviation roughly scales with the mean.

VIII. Discussion

Counting protein copy numbers *in vivo* using genetically encoded fluorescent proteins is a relatively simple but powerful approach. Its judicious use can potentially reveal critical information about subcellular structures as well as protein stoichiometry. This is especially true for many lower eukaryotes such as fungi and prokaryotes that are amenable to easy genetic manipulation for expressing genetically encoded fluorescent proteins. We have demonstrated the use of this method for understanding a key aspect of the molecular architecture of the kinetochore-microtubule attachment by counting the number of copies of protein complexes involved in linking centromeric DNA to a microtubule plus-end. Similar quantitative fluorescence microscopy assays can be extremely useful in establishing the lower eukaryotes as a platform for studying basic biological machinery that is conserved in all eukaryotes.

The use of similar techniques in studying vertebrate cell lines faces two challenges. The expression of fluorescently labeled proteins in vertebrate cells is commonly driven by an artificial promoter, in addition to the endogenous protein. As a result, the cells contain two protein species. More importantly, the fluorescently labeled protein is expressed at levels that can be significantly higher than the endogenous protein levels. With two species of the same protein within the cell, one protein type may get incorporated into the cellular process preferentially over the fusion protein. The interpretation of the experimental measurements

thus can be a complicated task in such cells. More importantly, these complications can deteriorate the accuracy achievable in counting protein numbers. The lack of suitable calibration standards is the second challenge if absolute protein numbers are desirable. Our work with the budding yeast centromere-specific histone protein Cse4p demonstrates that yeast cells expressing Cse4p-GFP can serve as a good fluorescent signal standard. Many other types of standards have been used in cell biology, and these are discussed below.

A. The Choice of Calibration Standards for Quantitative Fluorescence Microscopy

The most intuitive method of converting the observed GFP signal into a number of molecules is to divide the total fluorescence signal from a cell or cell organelle by the single molecule GFP fluorescence. Accurate determination of the fluorescence signal from a single GFP molecule, however, can be difficult. There are technical as well as practical issues with this approach. Evaluation of single molecule GFP fluorescence typically requires a highly sensitive imaging and image acquisition setup. While the fluorescence signal from single fluorophores very close to the coverslip can be accurately evaluated, it cannot be directly used as the reference signal for converting intracellular fluorescence into a number of molecules. More importantly, *in vitro* single molecule fluorescence properties such as quantum efficiency may differ from those *in vivo*.

A number of alternative methods have been developed to obtain an accurate calibration standard. The simplest standard is reconstituted GFP in solutions of known concentrations (Hirschberg *et al.*, 1998). The fluorescence signal obtained from such a standard is used as the reference for comparing the measured intracellular signal. The error in the measured number of molecules scales as the square of the average number of proteins in solution. Furthermore, the *in vitro* signal may differ from the intracellular GFP signal because of different excitation strength and quantum efficiency.

Another method was the use of virus capsids incorporating a GFP-fusion capsid protein (Dundr *et al.*, 2002). Because of the crystal-like structure of the shell, each individual shell incorporates a precise number of each of the constituent proteins including the GFP-fusion protein. Individual virus particles introduced/expressed in the cell can therefore be used to determine the single molecule GFP fluorescence intracellular environments.

Wu and Pollard (2005) used quantitative immunoblotting of GFP-fusion constructs in conjunction with quantitative fluorescence microscopy to construct a standard curve that can be used for converting the fluorescence signal from the entire cell for any other protein into a molecular count. This method directly links the measured fluorescence signal for a protein with biochemical determination of the concentration of the same protein via quantitative immunoblotting.

Recently, Rosenfeld *et al.* (2005, 2006) developed an ingenious method for converting the fluorescence signal into the number of fluorophores by making use of transiently expressed fluorescent proteins in dividing bacteria. A fixed number of proteins in a small dividing cell will be randomly partitioned into the daughter cells with a binomial distribution for the number of molecules partitioned in each cell.

Yet another possible approach is to use continuous photobleaching of a small number of closely clustered GFP molecules to resolve a step-wise decrease in the total fluorescence signal from the cluster (Leake *et al.*, 2006; Watanabe and Mitchison, 2002). These steps correspond to individual GFP molecules lapsing into a nonfluorescent state. This technique, however, requires a highly sensitive, low-noise image acquisition system, a small number of GFP molecules in a diffraction limited volume, and a careful selection of excitation and image acquisition conditions to be successful.

Advanced methods such as fluorescence correlation spectroscopy are particularly geared for measuring intracellular protein concentration of soluble proteins (Schwille, 2001). These methods, however, require specialized setups as well as expertise to acquire and analyze images to obtain concentration measurements.

B. Counting Protein Numbers from Volumes Larger than the Diffraction Limit

The kinetochore proteins in budding yeast are distributed over a subdiffraction volume. The technique described in this chapter focuses on ratiometric measurement of protein numbers by using the fluorescence from kinetochore clusters with Cse4p-GFP as the reference. More general instances of protein distribution in a cell include objects that are larger than the diffraction limit of the objective or proteins that are diffusely distributed over the volume of the cell. The nature of protein distribution along with the intended reference standard being used must both be considered in devising a suitable methodology for signal measurement. In either case, the extent of the spatial distribution of fluorescence signal intensity is a function of the actual dimensions of the object and the PSF of the objective. Especially for objects that are much larger than the diffraction-limit along the optical axis, it is important to use either a confocal microscope or a wide-field microscope with suitable deconvolution to assign the out-of-focus light to the correct plane. This has been described previously in Swedlow *et al.* (2002) using 6 μm fluorescent beads. Alternatively, confocal microscopy may also be useful as discussed later.

Accurate quantification of diffusely distributed proteins in thick specimens is best carried out using a confocal microscope. Measurements carried out with high NA objectives become susceptible to spherical and chromatic aberrations making it difficult to collect all the emitted light from molecules dispersed throughout the cell. Wu and Pollard (2005) made simultaneous use of fluorescence microscopy and quantitative immunoblotting of a set of candidate proteins to obtain a “standard curve” that relates measured fluorescence signal to a protein concentration value. Fluorescence signal was measured integrating the signal over the entire volume of the cell from a stack of images for each cell. To avoid deconvolution, Wu and Pollard used a Z step size that is equal to the full width at half maximum (FWHM) of the PSF of the objective along the optical axis. The assumption thus made is that the illumination intensity is uniform over this volume along the optical axis and over the entire thickness of the specimen. After establishing the cell boundaries in differential interference contrast (DIC) images, the fluorescence within these boundaries was integrated through all the image planes. The mean integrated signal for a given protein was then converted into an intracellular concentration by using the standard curve. The accuracy of this technique depends on the errors arising from immunoblotting, the errors in estimating protein content per cell from the total protein extracted for immunoblotting. These errors must be carefully minimized and evaluated in order to impose limits on the accuracy of the molecular counts.

C. Sources of Error in Fluorescence Signal Measurement

Variations in the number of protein obtained from different cells arise from a combination of experimental errors and inherent biological variability. It is therefore important to characterize the contribution of experimental errors so that the nature and reasons for biological variance can be studied. This is especially relevant in the study of the stochasticity in gene expression that is the major goal of fluorescence quantification experiments performed with prokaryotes and simple eukaryotes such as budding yeast (Raser and O’Shea, 2004; Rosenfeld *et al.*, 2005). As discussed earlier, the variance in the kinetochore protein number in each cluster is very low (less than 1 molecule per kinetochore). We also carried out fluorescence recovery after photobleaching (FRAP) experiments to demonstrate that there is no measurable turnover of kinetochore proteins (Joglekar *et al.*, 2006). Together with the low variance of protein numbers from cell to cell, the absence of protein turnover strongly suggests that the protein

assemblage at the kinetochore is built from a specific number of proteins that have a specific arrangement within the kinetochore structure.

Each step in the imaging, image acquisition, and data analysis contributes to the observed errors in the fluorescence signal. The relative contribution of each error must therefore be characterized and minimized. Possible sources in signal variation start from the excitation intensity variations. Mercury arc lamps generally emit a steady intensity, but it may vary over long periods of time. Also, misalignment of the lamp can result in significant changes in the excitation intensity. Commonly used laser sources in confocal microscopy show short- and long-term variations (Swedlow *et al.*, 2002). Periodic alignment of the excitation source and calibration of the excitation intensity is therefore important. Comparative fluorescence measurements are not sensitive to long-term changes in intensity variation. Auto-fluorescent background in cells is another common source of error. The relative magnitude and spatial variation in the background within the cell both limit the accuracy of measurements. The background light is usually the limiting factor that decides the lowest number of molecules that can be counted within the cell.

There are two major sources of noise that originate in the process of image acquisition: (1) shot noise arising from the photon counting statistics and (2) electronic noise due to the camera electronics. With the low-noise electronics used in modern CCD cameras, the contribution of electronic noise is typically miniscule as compared to other sources of error. The low-noise, back-thinned CCD cameras are also finding more and more use in cell biology. These cameras allow up to 90% photon conversion efficiency, thus significantly improving the signal for the same excitation intensity. The added efficiency is also useful in live-cell microscopy, since it allows the use of lower exposure times for the same sample reducing photobleaching and phototoxicity.

Finally, approximation of the extent of the Gaussian intensity spread, and measuring it with an array of square pixels also introduces errors in signal measurement (Joglekar *et al.*, 2006). The simplest way of measuring a signal is to draw a box centered on a pixel with the maximum signal value (which approximates the centroid of a symmetric spot). This procedure assumes, however, that the object centroid is approximately aligned with the center of a pixel in the CCD array. In reality, positioning of the object centroid is random over any given pixel. As a result, a box drawn with the brightest pixel as the center will on average result in clipping of the signal area. This error can be avoided for diffraction-limited spots by using Gaussian curve-fitting or cross-correlation algorithms to determine the exact centroid of the spot, and using the centroid location to determine the boundaries of the spot. It should be noted that ratio measurements are relatively insensitive to this error.

IX. Conclusions

Determination of fluorophore numbers from the total fluorescence signal measurement is one of the most immediate uses of fluorescence microscopy. With the focus of cell biology experiments shifting on a quantitative, mechanistic characterization of cellular functions, quantitative fluorescence microscopy makes it important to understand the organization of proteins within a protein assemblage. Quantitative fluorescence microscopy provides a new approach to studying protein organization *in vivo* by accurately counting the number of copies of each protein within the assemblage. Accurate protein counts along with nanometer-scale localization data can provide a level of detail that is otherwise not accessible with conventional methods.

Acknowledgments

A.P.J. holds a Career Award at the Scientific Interface from Burroughs-Wellcome Fund. This work was supported by NIH GM32238 to K.S.B. and NIH GM60678, NIH GM24364 to E.D.S.

References

- Agard DA, Hiraoka Y, Shaw P, Sedat JW. Fluorescence microscopy in three dimensions. *Methods Cell Biol* 1989;30:353–377. [PubMed: 2494418]
- Bar-Even A, Paulsson J, Maheshri N, Carmi M, O'Shea E, Pilpel Y, Barkai N. Noise in protein expression scales with natural protein abundance. *Nat. Genet* 2006;38:636–643. [PubMed: 16715097]
- Bloom KS, Beach DL, Maddox P, Shaw SL, Yeh E, Salmon ED. Using green fluorescent protein fusion proteins to quantitate microtubule and spindle dynamics in budding yeast. *Methods Cell Biol* 1999;61:369–383. [PubMed: 9891324]
- Chudakov DM, Lukyanov S, Lukyanov KA. Fluorescent proteins as a toolkit for *in vivo* imaging. *Trends Biotechnol* 2005;23:605–613. [PubMed: 16269193]
- Collins KA, Furuyama S, Biggins S. Proteolysis contributes to the exclusive centromere localization of the yeast Cse4/CENP-A histone H3 variant. *Curr. Biol* 2004;14:1968–1972. [PubMed: 15530401]
- Colman-Lerner A, Gordon A, Serra E, Chin T, Resnekov O, Endy D, Pesce CG, Brent R. Regulated cell-to-cell variation in a cell-fate decision system. *Nature* 2005;437:699–706. [PubMed: 16170311]
- Dundr M, McNally JG, Cohen J, Misteli T. Quantitation of GFP-fusion proteins in single living cells. *J. Struct. Biol* 2002;140:92–99. [PubMed: 12490157]
- Ghauharali RI, Brakenhoff GJ. Fluorescence photobleaching-based image standardization for fluorescence microscopy. *J. Microsc* 2000;198(Pt 2):88–100. [PubMed: 10810004]
- Gibson SF, Lanni F. Experimental test of an analytical model of aberration in an oil-immersion objective lens used in three-dimensional light microscopy. *J. Opt. Soc. Am. A* 1992;9:154–166. [PubMed: 1738047]
- Giepmans BN, Adams SR, Ellisman MH, Tsien RY. The fluorescent toolbox for assessing protein location and function. *Science* 2006;312:217–224. [PubMed: 16614209]
- Hiraoka Y, Sedat JW, Agard DA. Determination of three-dimensional imaging properties of a light microscope system. Partial confocal behavior in epifluorescence microscopy. *Biophys. J* 1990;57:325–333. [PubMed: 2317554]
- Hirschberg K, Miller CM, Ellenberg J, Presley JF, Siggia ED, Phair RD, Lippincott-Schwartz J. Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells. *J. Cell Biol* 1998;143:1485–1503. [PubMed: 9852146]
- Hoffman DB, Pearson CG, Yen TJ, Howell BJ, Salmon ED. Microtubule-dependent changes in assembly of microtubule motor proteins and mitotic spindle checkpoint proteins at PtK1 kinetochores. *Mol. Biol. Cell* 2001;12:1995–2009. [PubMed: 11451998]
- Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK. Global analysis of protein localization in budding yeast. *Nature* 2003;425:686–691. [PubMed: 14562095]
- Inoue, S.; Springer, KR. *Video Microscopy: The Fundamentals*. New York: Plenum; 1997.
- Joglekar AP, Bouck DC, Molk JN, Bloom KS, Salmon ED. Molecular architecture of a kinetochore-microtubule attachment site. *Nat. Cell Biol* 2006;8:581–585. [PubMed: 16715078]
- Kohl T, Schwill P. Fluorescence correlation spectroscopy with autofluorescent proteins. *Adv. Biochem. Eng. Biotechnol* 2005;95:107–142. [PubMed: 16080267]
- Kremers GJ, Goedhart J, van Munster EB, Gadella TW Jr. Cyan and yellow super fluorescent proteins with improved brightness, protein folding, and FRET Forster radius. *Biochemistry* 2006;45:6570–6580. [PubMed: 16716067]
- Kues T, Peters R, Kubitscheck U. Visualization and tracking of single protein molecules in the cell nucleus. *Biophys. J* 2001;80:2954–2967. [PubMed: 11371468]
- Leake MC, Chandler JH, Wadhams GH, Bai F, Berry RM, Armitage JP. Stoichiometry and turnover in single, functioning membrane protein complexes. *Nature* 2006;443:355–358. [PubMed: 16971952]
- Lippincott-Schwartz J, Patterson GH. Development and use of fluorescent protein markers in living cells. *Science* 2003;300:87–91. [PubMed: 12677058]

- Lippincott-Schwartz J, Snapp E, Kenworthy A. Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol* 2001;2:444–456. [PubMed: 11389468]
- Longtine MS, McKenzie A, Demarini DJ 3rd, Shah NG, Wach A, Brachet A, Philippsen P, Pringle JR. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 1998;14:953–961. [PubMed: 9717241]
- McAinsh AD, Tytell JD, Sorger PK. Structure, function, and regulation of budding yeast kinetochores. *Annu. Rev. Cell Dev. Biol* 2003;19:519–539. [PubMed: 14570580]
- Meluh PB, Yang P, Glowczewski L, Koshland D, Smith MM. Cse4p is a component of the core centromere of *Saccharomyces cerevisiae*. *Cell* 1998;94:607–613. [PubMed: 9741625]
- Meraldi P, McAinsh AD, Rheinbay E, Sorger PK. Phylogenetic and structural analysis of centromeric DNA and kinetochore proteins. *Genome Biol* 2006;7:R23. [PubMed: 16563186]
- Miyawaki A. Fluorescent proteins in a new light. *Nat. Biotechnol* 2004;22:1374–1376. [PubMed: 15529159]
- Miyawaki A, Nagai T, Mizuno H. Engineering fluorescent proteins. *Adv. Biochem. Eng. Biotechnol* 2005;95:1–15. [PubMed: 16080263]
- Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol* 2002;20:87–90. [PubMed: 11753368]
- Newman JR, Ghaemmaghami S, Ihmels J, Breslow DK, Noble M, DeRisi JL, Weissman JS. Single-cell proteomic analysis of *S. cerevisiae* reveals the architecture of biological noise. *Nature* 2006;441:840–846. [PubMed: 16699522]
- O'Toole ET, Winey M, McIntosh JR. High-voltage electron tomography of spindle pole bodies and early mitotic spindles in the yeast *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 1999;10:2017–2031. [PubMed: 10359612]
- Patterson GH, Knobel SM, Sharif WD, Kain SR, Piston DW. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J* 1997;73:2782–2790. [PubMed: 9370472]
- Piston DW. Choosing objective lenses: The importance of numerical aperture and magnification in digital optical microscopy. *Biol. Bull* 1998;195:1–4. [PubMed: 9739546]
- Piston DW, Patterson GH, Knobel SM. Quantitative imaging of the green fluorescent protein (GFP). *Methods Cell Biol* 1999;58:31–48. [PubMed: 9891373]
- Raser JM, O'Shea EK. Control of stochasticity in eukaryotic gene expression. *Science* 2004;304:1811–1814. [PubMed: 15166317]
- Raser JM, O'Shea EK. Noise in gene expression: Origins, consequences, and control. *Science* 2005;309:2010–2013. [PubMed: 16179466]
- Rosenfeld N, Perkins TJ, Alon U, Elowitz MB, Swain PS. A fluctuation method to quantify *in vivo* fluorescence data. *Biophys. J* 2006;91:759–766. [PubMed: 16648159]
- Rosenfeld N, Young JW, Alon U, Swain PS, Elowitz MB. Gene regulation at the single-cell level. *Science* 2005;307:1962–1965. [PubMed: 15790856]
- Salmon ED, Shaw SL, Waters J, Waterman-Storer CM, Maddox PS, Yeh E, Bloom K. A high-resolution multimode digital microscope system. *Methods Cell Biol* 2003;72:185–216. [PubMed: 14719333]
- Sawano A, Miyawaki A. Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis. *Nucleic Acids Res* 2000;28:E78. [PubMed: 10931937]
- Schwille P. Fluorescence correlation spectroscopy and its potential for intracellular applications. *Cell Biochem. Biophys* 2001;34:383–408. [PubMed: 11898862]
- Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol* 2004;22:1567–1572. [PubMed: 15558047]
- Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat. Methods* 2005;2:905–909. [PubMed: 16299475]

- Swedlow JR, Hu K, Andrews PD, Roos DS, Murray JM. Measuring tubulin content in *Toxoplasma gondii*: A comparison of laser-scanning confocal and wide-field fluorescence microscopy. *Proc. Natl. Acad. Sci. USA* 2002;99:2014–2019. [PubMed: 11830634]
- Taylor DL, Salmon ED. Basic fluorescence microscopy. *Methods Cell Biol* 1989;29:207–237. [PubMed: 2643761]
- Tsien RY. The green fluorescent protein. *Annu. Rev. Biochem* 1998;67:509–544. [PubMed: 9759496]
- Watanabe N, Mitchison TJ. Single-molecule speckle analysis of actin filament turnover in lamellipodia. *Science* 2002;295:1083–1086. [PubMed: 11834838]
- Wei RR, Sorger PK, Harrison SC. Molecular organization of the Ndc80 complex, an essential kinetochore component. *Proc. Natl. Acad. Sci. USA* 2005;102:5363–5367. [PubMed: 15809444]
- Winey M, Mamay CL, O'Toole ET, Mastronarde DN, Giddings TH Jr, McDonald KL, McIntosh JR. Three-dimensional ultrastructural analysis of the *Saccharomyces cerevisiae* mitotic spindle. *J. Cell Biol* 1995;129:1601–1615. [PubMed: 7790357]
- Wu JQ, Pollard TD. Counting cytokinesis proteins globally and locally in fission yeast. *Science* 2005;310:310–314. [PubMed: 16224022]
- Xie XS, Yu J, Yang WY. Living cells as test tubes. *Science* 2006;312:228–230. [PubMed: 16614211]
- Yu J, Xiao J, Ren X, Lao K, Xie XS. Probing gene expression in live cells, one protein molecule at a time. *Science* 2006;311:1600–1603. [PubMed: 16543458]
- Zhang J, Campbell RE, Ting AY, Tsien RY. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell Biol* 2002;3:906–918. [PubMed: 12461557]
- Zwier JM, Van Rooij GJ, Hofstra JW, BrakenhoV GJ. Image calibration in fluorescence microscopy. *J. Microsc* 2004;216:15–24. [PubMed: 15369479]

Fig. 1.

Use of quantitative fluorescence microscopy for understanding protein structure. The yeast kinetochore is a complex protein structure composed of 10 different proteins and protein complexes. From each complex, the constituent protein that was selected as the representative is indicated in green. Understanding protein architecture requires protein numbers and their relative localization within the kinetochore. Quantitative fluorescence microscopy can provide protein counts necessary for visualizing the three-dimensional (3-D) arrangement of different protein complexes. (See Plate no. 17 in the Color Plate Section.)

Fig. 2.

Distribution of kinetochores in a budding yeast cell in metaphase. (A) An image overlay of a metaphase cell expressing Cse4p-GFP to visualize the kinetochores and Spc29-RFP, which is a spindle pole body protein. (B) Cartoon of a metaphase budding yeast cell. Sixteen sister kinetochores cluster on either side of the spindle equator in a subdiffraction volume. Each kinetochore stably binds the plus-end of only one microtubule. (C) Dimensions of a typical metaphase and telophase spindle measured from the fluorescence intensity distribution agree with direct measurements of average kinetochore microtubule lengths using serial section electron microscopy (O'Toole *et al.*, 1999; Winey *et al.*, 1995). (See Plate no. 18 in the Color Plate Section.)

Fig. 3.

Fluorescence signal for different kinetochore proteins in metaphase and anaphase cells. A visual examination of the apparent intensity for different kinetochore proteins indicates that these proteins must be present in different numbers within the kinetochore cluster. The fluorescence signal from Cse4p-GFP provides an excellent calibration standard, as the number of Cse4p molecules per kinetochore, and hence the total number of GFP molecules in a cluster is known.

Fig. 4. Characterization of the intensity distribution from a kinetochore cluster. (A) The kinetochore clusters are positioned at random depths from the coverslip surface. Therefore, Z-stacks must be obtained by stepping the stage with an appropriate step size with respect to the objective. (B) The intensity distribution for a kinetochore clusters from cells expressing GFP-tagged kinetochore proteins (Nuf2p-GFP and Ndc80p-GFP) in the XY and XZ plane are shown. (C) The characteristic dimensions for the intensity distribution for a kinetochore cluster in telophase (●) are obtained by fitting it with a Gaussian function. The standard deviation obtained from the fit is similar to that for 200 nm beads (□). The theoretical PSF based on the objective NA (1.4) and wavelength (510 nm) is also plotted (solid curve). The Z intensity

distribution allowed us to determine the optimal step size along the Z axis (● for a kinetochore cluster and □ for a 200 nm bead).

Fig. 5.

Linear response of the technique for measuring the fluorescence signal from diffraction-limited images of kinetochore clusters in telophase. (A) The doubling of the kinetochore proteins that have been tagged with GFP is accurately reflected by a doubling of the measured fluorescence signal. This validates our ratiometric method for counting the number of molecules within a kinetochore cluster. (B) Single kinetochores can be imaged in a cell expressing Nuf2p-GFP after the activation of the dicentric chromosome. (See Plate no. 19 in the Color Plate Section.)

Fig. 6. Decrease in signal with increasing depth from the coverslip surface. Because of spherical aberrations, the signal measured from a kinetochore cluster decreases as a function of the distance from the coverslip surface. This decrease is independent of the magnitude of the signal (●: Cse4p-GFP; ○: Nuf2p-GFP+Ndc80p-GFP).

Characteristics Selection of Optimal Fluorescent Proteins for Fluorescence Quantification Experiments

Table 1

Protein	Excitation wavelength ^a (nm)	Emission wavelength ^a (nm)	Brightness ^a	<i>t</i> _{1/2} maturation time (min) ^a	Photostability ^a
Cerulean	433	475	27	<5 ^b	36
EGFP	488	507	34	<5	174
EYFP	514	527	51	<5 ^c	60
mCherry	587	610	16	15 ^d	96

^aShaner *et al.* (2005) provides a comprehensive compilation of all the important characteristics of the available fluorescent proteins. *t*_{1/2} as estimated by completely denaturing the protein *in vitro*, and then measuring the fluorescence recovery half-life as the protein renatures.

^bKremers *et al.* (2006).

^cNagai *et al.* (2002).

^dShaner *et al.* (2004).

Kinetochores Protein Counts

Table II

Complex	Protein	Vertebrate homolog	Ratio		Number	
			Metaphase	Anaphase	Metaphase	Anaphase
Nucleosome	Cse4p	CENP-A	1	1	2	2
	Mif2p*	CENP-C	5.4 ± 0.4	5.5 ± 0.1	1-2	1-2
	Ndc10p	-	1.9 ± 0.2	1.3 ± 0.01	4	2-3
CBF3	Cep3p	-	0.9 ± 0.2	0.6 ± 0.01	2	1-2
COMA	Ctf19p*	CENP-F	3.4 ± 0.3	3.4 ± 0.2	3	2
	Spc105p	KNL-1, AF15q14	2.4 ± 0.01	2.4 ± 0.01	5	5
MIND	Mtw1p	Mis12, CENP-H	3.3 ± 0.2	2.4 ± 0.1	6-7	4-5
NDC80	Nuf2p	Nuf2	4.0 ± 0.2	3.4 ± 0.05	8	7
DAM/DASH	Ask1p	xDam1p?	9.0 ± 1.0	5.3 ± 0.3	16-20	10-12

The table lists the average ratio of the average signal from the protein of interest with that for Cse4p-GFP from three experiments. Cells from two strains in each experiment were distinguished based on the magnitude of the fluorescence signal. This approach could not be used for Mif2p and Ctf19p, as the intensities for these proteins were very similar to that for Cse4p-GFP making manual identification of cells impossible. For these proteins, we used the fluorescence signal from cells expressing Nuf2p-GFP as the reference. The ratios reported for these proteins are Nuf2p-GFP:protein of interest.

Table III

Analysis of the Observed Variance

	Cse4p-GFP	Nuf2p-GFP	Nuf2p-GFP + Ndc80p-GFP
<i>N</i> (clusters)	90	104	104
Average	1945	6796	15523
Standard deviation	428	1987	2773
Clusters separated by less than 400 nm			
<i>N</i> (cells)	21	13	23
Average difference	240	840	1195
SEM based on average difference	45	232	249

Decrease in the measured signal as a function of the depth at which signal measurement is carried out contributes significantly to the observed variance in the signal. By minimizing its contribution, we find that the biological variance in the number of kinetochore proteins from cell to cell is very low.