Every laboratory with a fluorescence microscope should consider counting molecules

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ABSTRACT **Protein numbers in cells determine rates of biological processes, influence the architecture of cellular structures, reveal the stoichiometries of protein complexes, guide in vitro biochemical reconstitutions, and provide parameter values for mathematical modeling. The purpose of this essay is to increase awareness of methods for counting protein molecules using fluorescence microscopy and encourage more cell biologists to report these numbers. We address the state of the field in terms of utility and accuracy of the numbers reported and point readers to references for details of specific techniques and applications.**

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INTRODUCTION

Biology has benefited tremendously from the application of quantitative techniques (reviewed in Mogilner *et al.*, 2012; Morelli *et al.*, 2012). Numbers of molecules, stoichiometries, and concentrations are important for making the most use of quantitative simulations and proposing structural models. In addition, these measurements are critical for in vitro reconstitution and other biochemical assays. The introduction of green fluorescent protein (GFP) and its variants into research laboratories across the globe revolutionized the way we study cells. The linear relationship of signal intensity to the number of GFP molecules reveals more than spatial and temporal information for GFP-tagged proteins. Researchers can count tagged proteins in a living or fixed cell if the fluorescence output of a single GFP molecule is determined.

Counting protein molecules by fluorescence microscopy only requires a fluorescence imaging system and some basic analysis tools such as ImageJ (National Institutes of Health, Bethesda, MD), a free download. Thus research laboratories commonly publishing microscopy data should take advantage of the availability of this technique. Using microscope images to report only qualitative data or even arbitrary units of fluorescence intensity is an underutilization of the data set, especially if the protein is endogenously tagged.

Previously yeast model organisms offered an advantage to anyone counting molecules because of the ability to tag endogenous genes using efficient homologous recombination. The recent rapid development of genome editing techniques using DNA or RNA nucleases targeted to specific sequences makes counting molecules possible in many other cell types (Coffman and Wu, 2012). Current genome editing techniques have been reviewed elsewhere (Ramalingam *et al.*, 2013; Wei *et al.*, 2013; Aida *et al.*, 2014; Chen and Gao, 2014; Mashimo, 2014). Although some of these technologies are still expensive and maturing, they are not absolutely necessary if one can measure the ratio of tagged and untagged protein in the cells and structure of interest (Engel *et al.*, 2009; Johnston *et al.*, 2010).

TECHNIQUES

The two most common techniques for measuring protein molecules by fluorescence microscopy are stepwise photobleaching to count steps and comparing the fluorescence intensity of a protein to a known standard. The specifics of these techniques are reported elsewhere (Wu and Pollard, 2005; Joglekar *et al.*, 2006; Leake *et al.*, 2006; Ulbrich and Isacoff, 2007; Sirotkin *et al.*, 2010; Coffman *et al.*, 2011; Laporte *et al.*, 2011). One advantage of the photobleaching method is that it does not require a standard, but the disadvantage is that it is useful only in a subset of cases, especially when the molecule numbers are relatively low (for discussion see Coffman and Wu, 2012). The ratio method is more broadly applicable, but there are differing opinions about the best way to carry it out. A sum of all *z*-sections with signal spaced appropriately (Hirschberg *et al.*, 1998; Wu and Pollard, 2005; Sirotkin *et al.*, 2010; Coffman *et al.*, 2011; Coffman and Wu, 2012; Laporte *et al.*, 2011) is necessary to measure all the protein in the cell, and we prefer this method for measurements of specific structures. Others used only the focal plane with maximum intensity for both the standard and the protein of

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Abbreviations used: CENP-A, centromere protein A; FCS, fluorescence correlation spectroscopy; GFP, green fluorescent protein; PALM, photoactivated localization microscopy.
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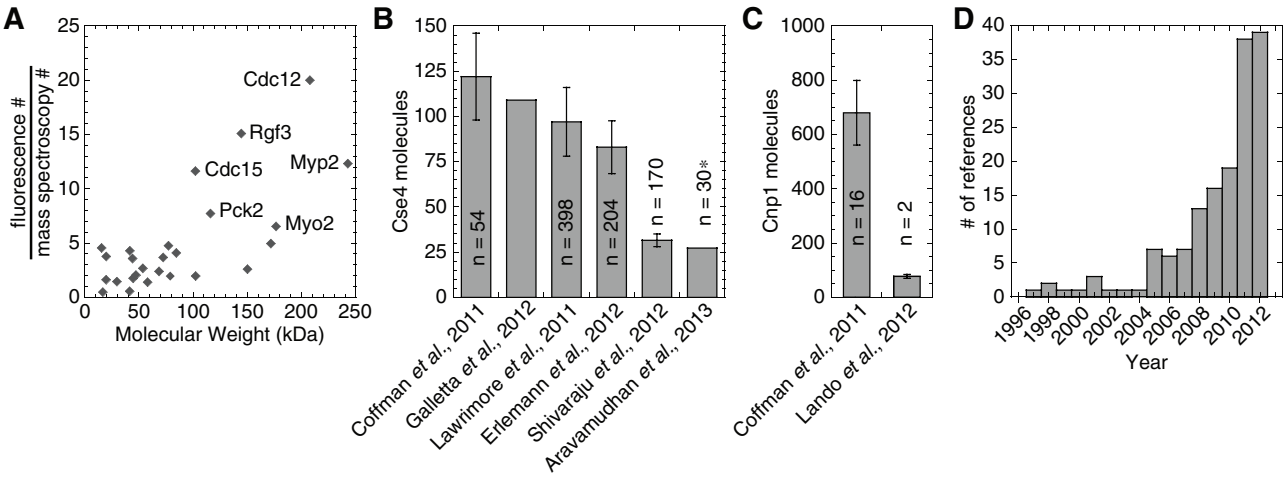

FIGURE 1: Comparisons of protein numbers counted by different quantification methods. (A) The quotient of the protein numbers in fission yeast cells from Table 1 of Wu and Pollard (2005) divided by the data from mass spectrometry for the same proteins (Marguerat *et al.*, 2012) plotted vs. the predicted molecular weights of the proteins (PomBase, www.pombase.org). The majority of quotients (20/26) are <5. Proteins with quotients >5 are labeled. (B) Comparison of fluorescence measurements of CENP-A Cse4 in anaphase clusters in *S. cerevisiae* using various standards and methods (Coffman *et al.*, 2011; Lawrimore *et al.*, 2011; Erlemann *et al.*, 2012; Galletta *et al.*, 2012; Shivaraju *et al.*, 2012; Aravamudhan *et al.*, 2013). Asterisk indicates that this number was measured indirectly, *n* is for Spc24 measurement, and the Cse4 number is given by the ratio comparison from Joglekar *et al.* (2006). (C) Comparison of fluorescence ratio measurement (Coffman *et al.*, 2011) to measurement by PALM (Lando *et al.*, 2012) for *S. pombe* CENP-A Cnp1 in anaphase clusters. (D) Histogram of the number of articles each year from 1996 to 2012 using fluorescence methods to count proteins. This is by no means an exhaustive tabulation, but it includes >100 cross-references from the key papers on the subject.

interest (Joglekar *et al.*, 2006), but we showed that this method does not always agree with the sum measurement, depending on the distribution of the signal in the *z*-direction (Coffman *et al.*, 2011). A change in size or shape of a structure during the cell cycle could contribute some error to the maximum-plane measurement. The ratio method is insensitive to the fluorophore used to some extent, because the same fluorescent protein fused to both the standard and protein of interest will have similar maturation efficiency, brightness, and other features (Coffman and Wu, 2012; Erlemann *et al.*, 2012).

There are two types of measurements that might be useful: global protein content in the cell and local protein concentration in a structure or location of interest. For global protein content, otherwise isogenic cells with no fluorescent protein serve as a background control to subtract out cellular autofluorescence, as well as any offset from the camera and system (Wu and Pollard, 2005). Global protein measurements are best done by comparing fluorescence intensities to immunoblotting or to proteins of known concentration using a linear curve. For local measurements, background subtractions remove the average cytoplasmic concentration from inside the region of interest while simultaneously accounting for autofluorescence and offset. We and others have used a concentric background region of interest or a similar-sized region from nearby (Hoffman *et al.*, 2001; Wu and Pollard, 2005; Joglekar *et al.*, 2006, 2008; Johnston *et al.*, 2010; Sirotkin *et al.*, 2010; Coffman *et al.*, 2011; Laporte *et al.*, 2011). The choice between these two depends on the proximity of other concentrated fluorescence. Local measurements can be taken using the photobleaching method for fewer molecules or a ratio method. Measurements in fission yeast are not obviously affected by quenching from local protein accumulation (Wu and Pollard, 2005; Coffman *et al.*, 2011; Coffman and Wu, 2012).

STANDARDS FOR COUNTING PROTEIN MOLECULES BY FLUORESCENCE MICROSCOPY

Several in vitro and in vivo standards for counting proteins have been reported and widely used. The most basic standard is the fluorescence of a single GFP molecule, which can be measured in several ways: 1) by determining loss of fluorescence intensity during stepwise photobleaching; 2) by directly measuring speckles in a dilute sample of purified GFP; and 3) by making a linear curve using known concentrations of purified GFP-tagged protein adjusted for the ratio of fluorescence of bulk beads to that of single beads (Leake *et al.*, 2006; Graham *et al.*, 2011; Lawrimore *et al.*, 2011). In fission yeast, numbers from fluorescence microscopy that agree with flow cytometry and immunoblotting have been reported up to ~10^5 molecules/cell (Wu and Pollard, 2005). Although GFP fluorescence is affected by its environment, measurements in different organisms and comparisons to in vitro GFP seem largely insensitive to differences in environment (Coffman and Wu, 2012). There are many considerations when choosing fluorescent tags and standards to use; these are reviewed elsewhere (Shaner *et al.*, 2005; Chudakov *et al.*, 2010; Coffman and Wu, 2012). The budding yeast homologue of centromere protein A (CENP-A) Cse4 has frequently been used as a counting standard, but problems have arisen (see later discussion). Thus the fluorescence of a single GFP molecule or a calibration curve composed of a range of molecule numbers is a more suitable standard.

ACCURACY OF FLUORESCENCE QUANTIFICATION

Two recent examples yield some useful insight into the accuracy of current methods for counting molecules by fluorescence microscopy. Cytokinesis proteins in the fission yeast *Schizosaccharomyces pombe* have been counted by live-cell fluorescence microscopy (Wu and Pollard, 2005) and by mass spectroscopy (Marguerat *et al.*, 2012). Comparison of these two data sets shows that 77% of the proteins fall below a fivefold difference (Figure 1A). It is important to

note that the growing conditions of the strains were different. Wu and Pollard (2005) used rich medium, whereas Marguerat *et al.* (2012) used minimal medium. There is a twofold reduction in actin concentration in minimal medium compared with rich medium (Wu and Pollard, 2005), which might explain the differences for most of the proteins. In addition, the proteins with numbers greater than fivefold higher in the fluorescence microscopy data set are all large proteins (>100 kDa), which might affect the accuracy of the mass spectroscopy data (Figure 1A). Indeed, we note that the formin Cdc12 is one such protein for which the fluorescence microscopy value is ~600 molecules/cell compared with ~30 in the mass spectroscopy data. Fluorescence microscopy shows that each cell has at least 200 speckles that are believed to be dimers (Coffman *et al.*, 2009), suggesting that the fluorescence microscopy data are more accurate. A recent estimate of total proteins per cell volume (Milo, 2013) indicates the mass spectroscopy data set might underestimate protein numbers by approximately fivefold, which is consistent with the fluorescence data for most of the proteins. Fluorescence microscopy measurements are less susceptible to error arising from protein size or abundance and therefore are likely to be more accurate than mass spectroscopy. Moreover, mass spectroscopy is not useful for counting local concentrations in most cases.

The second example that we would like to highlight is the disagreement over measurements of centromere-specific protein CENP-A in budding and fission yeast. The *Saccharomyces cerevisiae* (budding yeast) CENP-A Cse4 counted by fluorescence microscopy ranges from 32 to 122 per anaphase cluster (Figure 1B) or 2 to ~8 per centromere, whereas chromatin immunoprecipitation (ChIP) data imply 2 Cse4 molecules per centromere. This is an important distinction, as it might affect structural models of the centromere and kinetochore and the definition of a point centromere. Two of the fluorescence measurements of Cse4 seem to support the number obtained by ChIP (Shivaraju *et al.*, 2012; Aravamudhan *et al.*, 2013), but Lawrimore *et al.* (2011) showed convincing evidence that ChIP does not yield accurate numbers of proteins bound to centromeric DNA due to its measurement of population averages. There are fewer measurements of the fission yeast CENP-A Cnp1, but ChIP data give a number that lies between the two fluorescence measurements (Figure 1C). Lawrimore *et al.* (2011) used the ratios reported in Joglekar *et al.* (2008) to adjust the *S. pombe* kinetochore numbers, but the tagged Cnp1 in Joglekar *et al.* (2008) was not the sole copy of Cnp1 (Coffman *et al.*, 2011; Yao *et al.*, 2013). Ndc80 numbers agree closely in three studies (Coffman *et al.*, 2011; Lawrimore *et al.*, 2011; McCormick *et al.*, 2013), suggesting that the photoactivated localization microscopy (PALM) measurement (Lando *et al.*, 2012) might be overcorrected to account for blinking. One possible explanation for the difference between ChIP and fluorescence measurements in both yeasts might be that not all CENP-As in anaphase clusters are associated with centromeric DNA (Haase *et al.*, 2013). In addition, the distribution of Cse4 at budding yeast centromere clusters is not consistent with only 2 molecules per centromere (Haase *et al.*, 2012, 2013). Thus further experiments are needed to determine the amount of CENP-A that contributes to centromere identity in both budding and fission yeasts (Maresca, 2013). However, even the largest and smallest numbers differ by only fourfold (Figure 1B), which might suffice for some applications. Until a consensus is reached, CENP-A proteins are not the best standards to use in fluorescence quantification. Fortunately, the calibration curves for budding (Lawrimore *et al.*, 2011) and fission (Wu and Pollard, 2005; McCormick *et al.*, 2013) yeasts are suitable for measuring protein numbers over several orders of magnitude.

SOURCES OF ERROR

Each method to count molecules has sources of error, and some methods are more technically demanding or require specialized analytical skills. Counting molecules by photobleaching requires a very sensitive imaging system, and the low signal-to-noise ratio introduces errors (Waters, 2009). Detecting the step boundaries in photobleaching data requires user-defined criteria and can be challenging since the data are usually noisy. The modified Chung–Kennedy algorithm was used to aid in defining step boundaries (Leake *et al.*, 2006; Engel *et al.*, 2009; Coffman and Wu, 2012), but the precise boundaries between plateaus might not always be obvious. By assembling a large data set of step sizes, one can attenuate the inaccuracy of defining step boundaries.

Ratio measurements require only a standard fluorescence microscope and a digital camera, but low signal-to-noise ratio is still a concern. Autofluorescence noise can also contribute to errors when GFP intensity is <1.5 times the autofluorescence (Heinrich *et al.*, 2013). The main challenge is the reliability of the standards used to convert fluorescence intensity directly into molecule numbers. A calibration curve is more accurate than a single standard, especially when measured proteins span several orders of magnitude (Wu and Pollard, 2005; McCormick *et al.*, 2013).

Two additional fluorescence microscopy methods not described in detail here have been used to count molecules in live cells. Fluorescence correlation spectroscopy (FCS; Shivaraju *et al.*, 2012) is an established method for determining concentrations of dilute fluorescent proteins in addition to single-molecule dynamics and mobility (Kim *et al.*, 2007). Because this determination is done within a defined volume, the number of molecules is calculable (Meyer and Schindler, 1988). FCS is particularly suited to quantifying molecular dynamics when fluorophores are at nanomolar concentration and are highly mobile. FCS is limited in its application for counting molecules because of its sensitivity to photobleaching and population heterogeneity (Kim *et al.*, 2007).

PALM, a superresolution microscopy technique, has recently emerged as a method to directly count molecules in live cells (Annibale *et al.*, 2011; Lando *et al.*, 2012; Sengupta and Lippincott-Schwartz, 2012). The basic idea of superresolution microscopy is to observe molecules one at a time so that their precise location can be determined. As a result, it should be possible to count molecules directly without the need for separate standards. Unfortunately, the analysis methods for PALM are still fraught with uncertainties, which make it difficult to produce accurate counts. The main difficulty is being able to count each molecule once and only once, partly because photoactivatable fluorescent proteins are able to blink on and off in subsequent images (Annibale *et al.*, 2011; Lando *et al.*, 2012; Sengupta and Lippincott-Schwartz, 2012). As analysis algorithms (Sengupta and Lippincott-Schwartz, 2012) and photoactivatable fluorescent proteins (Zhang *et al.*, 2012) improve, PALM could eventually become the gold standard for counting molecules.

CONCLUSION

Based on the increasing number of articles reporting protein numbers by fluorescence microscopy in the past two decades (Figure 1D), this technique has proven to be useful. Stoichiometries of the budding yeast kinetochore (Joglekar *et al.*, 2006), budding yeast γ-tubulin ring complex (Erlemann *et al.*, 2012), fission yeast cytokinesis node (Laporte *et al.*, 2011), fission and budding yeast endocytic patches (Sirotkin *et al.*, 2010; Galletta *et al.*, 2012), bacterial replisome (Reyes-Lamothe *et al.*, 2010), and many other complexes have been elucidated based on these methods, which are essential for proposing structural models. The next step in many of these cases is

to use the stoichiometric data to inform and constrain in vitro reconstitution experiments and mathematical models of the function or assembly mechanisms of these complexes.

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