Every laboratory with a fluorescence microscope should consider counting molecules

Valerie C. Coffman* and Jian-Qiu Wu**
*Department of Molecular Genetics and **Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH 43210

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.

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 any-one 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
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., 2011; Joglekar et al., 2006; Graham 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 ~10^3 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 fluorochrome 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 spectrometry (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-A 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 (Erlmann et al., 2012), fission yeast cytosome 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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