FPCountR: Absolute quantification of fluorescent proteins for synthetic biology

Eszter Csibra and Guy-Bart Stan

Department of Bioengineering, Imperial College Centre for Synthetic Biology (IC-CSynB), Imperial College London, London, UK, SW7 2AY

Correspondence: Eszter Csibra (e.csibra@imperial.ac.uk) and Guy-Bart Stan (g.stan@imperial.ac.uk)

Keywords: absolute quantification, calibration, engineering biology, fluorescent proteins, microplate reader, synthetic biology

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; EC, extinction coefficient; EDTA, ethylenediaminetetraacetic acid; FP, fluorescent protein, GFP, green fluorescent protein, MEFL, molecules of equivalent fluorescein, MEFP, molecules of equivalent fluorescent protein, OD, optical density; PEMS, particles of equivalent microspheres, SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEVA, Standardised European Vector Architecture
Abstract

This paper presents a generalisable method for the calibration of fluorescence readings on microplate readers, in order to convert arbitrary fluorescence units into absolute units.

FPCountR relies on the generation of bespoke fluorescent protein (FP) calibrants, assays to determine protein concentration and activity, and a corresponding analytical workflow. We systematically characterise the assay protocols for accuracy, sensitivity and simplicity, and describe a novel ‘ECmax’ assay that outperforms the others and even enables accurate calibration without requiring the purification of FPs. To obtain cellular protein concentrations, we consider methods for the conversion of optical density to either cell counts or alternatively to cell volumes, as well as examining how cells can interfere with protein counting via fluorescence quenching, which we quantify and correct for the first time. Calibration across different instruments, disparate filter sets and mismatched gains is demonstrated to yield equivalent results. It can also reveal that mCherry absorption at 600nm does not confound cell density measurements unless expressed to over 100,000 proteins per cell. FPCountR is presented as pair of open access tools (protocol and R package) to enable the community to use this method, and ultimately to facilitate the quantitative characterisation of synthetic microbial circuits.
Introduction

There is a growing awareness that tackling the challenge of synthetic circuit design requires the synthesis of empirical characterisation data on genetic parts with mathematical modelling approaches for predicting and realising desired behaviours (Delvigne et al., 2018; Beal et al., 2020; Perrino et al., 2021). However, there are numerous challenges in integrating experimental data with quantitative frameworks, as experimental data is typically acquired in relative or arbitrary units (specific to instruments and their respective settings), which cannot be converted into useful units and therefore limits our ability to make comparisons between experiments and models.

Fluorescent proteins (FPs) are our most versatile tools for the assessment of synthetic genetic element performance. Since their discovery, FPs have rightly been recognised as uniquely valuable reporter proteins for quantitative characterisation (Chalfie et al., 1994; Rodriguez et al., 2017), since they do not require the addition of exogenous components to fluoresce. This makes their use easy and cost effective. Various laboratory instruments allow the characterisation of fluorescent systems in a wide range of dimensions and scales – through direct visualisation (using fluorescence microscopy), via single-cell fluorescence analysis (using flow cytometry), or via timecourse kinetic data acquisition (using microplate readers).

The ‘protein quantification problem’ of reporting GFP levels acquired by such instruments in ‘relative fluorescent units’ (RFU) has been widely recognised in synthetic biology (Beal et al., 2018). This recognition has led to the adoption of calibration standards such as fluorescein,
a small molecular fluorophore with similar excitation and emission characteristics to GFP.

Fluorescein can be used to calibrate a given instrument by converting the instrument’s arbitrary RFU output into units of ‘molecules of equivalent fluorescein’ (MEFL). This technique has been demonstrated to enable the comparison of GFP expression data gathered from different instruments (Fedorec et al., 2020) as well as across laboratories (Beal et al., 2018).

While it is now approaching mainstream usage in synthetic biology, the conversion of green fluorescence values into MEFL is arguably not the most important type of quantification required for building synthetic circuits. Three aspects of the protein quantification problem remain elusive. First, fluorescein is only a good calibrant for green fluorescent proteins, leaving blue, yellow, orange and red FPs uncalibrated. Second, fluorescein can only provide a conversion to units of fluorescein, whereas what is actually needed is a conversion to units of protein. Currently, most experiments cannot even reveal the order of magnitude at which FPs are being expressed (ie. 10 molecules per cell vs 100,000) – in contrast to what is possible with RNA sequencing (Gorochowski et al., 2019) – which limits our ability to understand how protein abundance contributes to cellular burden (Ceroni et al., 2015; Boo et al., 2019). Third, while fluorescein can allow the comparison of GFP levels between instruments and laboratories, it cannot address the comparison between two different FPs in the same circuit. This is only possible if RFUs from both FPs can be separately converted into molecular units of protein. Some have attempted to tackle this by attempting to predict the relative brightness of FPs using theoretical values (Boada et al., 2019; Vignoni et al., 2019), but such calibrations make a number of unvalidated assumptions, for instance about
translation rate equivalence across constructs, that require rigorous testing before they can be adopted.

Fortunately, there is a reasonably simple solution. The ideal calibrant in molecular biology is considered to be a purified sample of the molecule to be measured – in this case, the fluorescent protein itself. While purified FPs are not commercially available, they can be produced ‘in-house’, thereby providing that crucial direct link between relative fluorescence units and molecules of protein. Indeed, others have proposed protocols for such calibrations (Taniguchi et al., 2010; Finan et al., 2015; Hirst et al., 2015; Tie et al., 2016), although such practice remains rare in the literature. We suspect this is due to (a) an underappreciation that absolute quantification is possible without ‘omics’, (b) a reticence to try unfamiliar biochemical protocols that are not usually part of the synthetic biology repertoire, and (c) doubt that such protocols could be accurate or sensitive enough for general usage. We suspected that many of these assertions were due to misunderstandings about protein purification methodologies and the biochemical characteristics of fluorescent proteins in general, and this motivated us to develop a general, yet simple-to-use calibration protocol of this nature.

In what follows, we outline our optimised calibration method and present it as a pair of resources: a wet lab protocol, called FPCount (available on protocols.io) and an accompanying analysis package, called FPCountR (available on GitHub). We present data showing the development of this protocol, and systematically characterise the biochemical and analytical requirements of an accurate and sensitive calibration. We also present a ‘hack’, which acts both to simplify the method to remove the requirement for protein
purification, and to make it more sensitive and robust. Using FPCountR, we show that conversion to molecular units can be used to calibrate across different instruments, disparate filter sets and mismatched gains to yield equivalent results. We demonstrate that conversion to absolute units allows the user of our method to compare the protein production efficiency of different fluorescent proteins expressed from an otherwise identical vector in molecules per cell, or as a molar concentration. Finally, we demonstrate that this method can be used to quantitatively evaluate the experimental protocols themselves, such as the extent to which red fluorescent proteins confound optical density readings in timecourse assays.

Results

Our aim for this work was to develop a generalisable method for FP calibration that could be used by any group wishing to calibrate fluorescence readings on microplate readers to molecular units. To do this, we defined a number of key aims for our proposed method. First, it should be accurate and sensitive, as we need the method to correctly estimate molecule numbers within cells, and as protein yields from small-scale purifications are typically modest. Second, the calibration protocol should be as simple as possible and adapted ideally such that each respective assay may be carried out in 96-microwell plate format using the same plate reader that is being calibrated. This way, multiple fluorescent proteins may be calibrated at once, and end users do not require any additional instrumentation. Third, the method should be suitable for the particular characteristics of fluorescent proteins. These proteins are smaller and structurally distinct from typical protein calibrants such as bovine serum albumin (BSA), and are known to present certain
challenges for quantification due to light absorption by their chromophores. Thus, any assay developed for non-fluorescent proteins requires a separate validation on FPs to demonstrate that they are also adequate for this class of proteins. Finally, we wanted to enable the easy analysis of the data, by (i) enabling easy conversion of raw calibration data into a conversion factor that links the arbitrary fluorescence output of a protein with its quantity in molecular units, and (ii) allowing easy conversion of data from all future timecourse data from that instrument to produce outputs in (e.g. GFP) molecules, rather than relative (e.g. green) fluorescence units. An overview of the FPCount fluorescent protein calibration protocol is illustrated in Fig. 1.

**Design of purification protocol for obtaining protein calibrants**

In order to obtain our fluorescent protein calibrants, they must first be produced by overexpression, and purified. Protein purification methodologies can often be highly complex, requiring specialist expertise and instrumentation. To alleviate this, our protocol was explicitly designed to be as straightforward as possible, involving only the minimum number of required steps, and using commonly available reagents. It was also designed to be amenable to small-volume purifications to enable the calibration of multiple proteins in parallel. The protocol is summarised in Fig. 2A. A standardised FP expression vector was constructed from an arabinose-inducible His-tagged FP construct in a high-copy SEVA vector (Fig. 2B). Overnight expression was used to maximise protein production, and cells were lysed using sonication to avoid the requirement to add chemical components that may interfere with downstream processes, such as EDTA (with His-tag purification) or detergents (with protein quantification). Insoluble proteins were removed via centrifugation and proteins were purified using cobalt resin to minimise the co-isolation of impurities.
Expression yields and solubility were checked by SDS-PAGE (Fig. 2C) and the quality of purified FP calibrants was verified by SDS-PAGE and fluorescence excitation and emission scanning (Fig. 2D-E, Supplementary Fig. 1). Purified calibrants were of consistently good purity and yield.

**Conducting a plate reader calibration**

The calibration of plate readers with fluorescein has traditionally been conducted using a dilution series of known concentrations of fluorescein, subjected to a fluorescence assay in the plate reader whose calibration is desired (measurement of relative fluorescence units, RFU). The results can be used to relate fluorescein molecule number to RFU to obtain a conversion factor, which can in turn be used to convert RFU readouts from experimental data into MEFL units (Beal et al., 2018). For protein calibrants, one additional step is required: protein concentration determination.

In our initial protocols, we opted for the bicinchoninic acid (BCA) assay due to its sensitivity, ease of use and low protein-to-protein variability (Noble et al., 2007). In addition, microplate-optimised reagents for a ‘microBCA’ were available from ThermoFisher, with excellent reported sensitivities (to 2 ng/µl). Pilot tests showed an inhibitory effect of the Tris and NaCl in the elution buffer (Supplementary Fig. 2A) suggesting that buffer exchange would be necessary for high assay sensitivity. Background signal was also observed from protease inhibitors in the buffer, but this didn’t seem to disrupt quantification accuracy.

Normalised BSA values were fitted to a polynomial equation to obtain a standard curve (Supplementary Fig. 2D, ii). This was then used to predict the concentrations of FP dilutions by first removing values under the reported threshold of sensitivity and fitting a linear
model through the rest of the values (Supplementary Fig. 2D, ii and iii). An extra step was added to the recommended protocol (Supplementary Fig. 2A, ii and Supplementary Fig. 2C) to account for baseline absorbance of red FPs in the A562 range. The calculations are handled by the `get_conc_bca()` function. Using these predicted protein concentrations, an adapted version of the `generate_cfs()` function from flopR (Fedorec et al., 2020) was used to generate conversion factors for mCherry in a Tecan Spark plate reader for the RFP-typical filter set (ex 560/20, em 620/20), showing that conversion factors from RFU to molecules may be obtained using the FPCount protocol analogously to obtaining conversion factors from RFU to MEFL for fluorescein. Example outputs are shown in Supplementary Fig. 3.

**Development of a second assay for FP concentration – the A280 assay**

We sought to verify the accuracy of the BCA assay by re-quantifying our FPs with a second method that is likely to give reliable concentration estimates. The most commonly used assays for protein quantification include SDS-PAGE-based Coomassie assays and Bradford assays (Bradford, 1976; Noble, 2014), both based on the same reaction, which has been reported to possess more protein-to-protein variability than the BCA assay (Tal et al., 1985; Noble et al., 2007; Thermo Fisher Scientific, 2017). It has also been suggested that proteomics could be used for absolute quantification. However, proteomic data is also not inherently ‘absolute’ – to obtain accurate absolute protein counts users typically need to provide a calibrant of known concentration that is treated identically to the sample (Wasinger et al., 2013; Calderón-Celis et al., 2018). Once again, the calibrant should ideally be identical to the protein we wish to count, i.e. purified FP. Using proteomics to validate our BCA results for our FP calibrants would therefore be a logically circular process that wouldn’t allow us to validate our method.
While a wide variety of protein assays exist, the only widely-used ‘absolute’ assay that does not require a calibrant is the A280 assay. As the name suggests, it quantifies protein concentration via light absorbance at 280 nm, where three amino acid residues are known to absorb light in a way that has been shown to be approximately additive (Pace et al., 1995). This finding means that a reasonable prediction of light absorbance at 280 nm can be made for any pure protein of known primary sequence by way of an extinction coefficient (EC; expected light absorption for a given concentration of protein). As sample absorbance relates to molecular concentration according to Beer’s law, i.e., \( A = EC \times C \times L \), (where \( A \) is the absorbance, \( EC \) is the extinction coefficient \( (M^{-1} cm^{-1}) \), \( C \) is the concentration \((M)\), and \( L \) is the path length \((cm))\), the protein concentration may be calculated from absorbance using only the extinction coefficient and the path length. The most common formats for A280 measurements are laborious, single-throughput cuvette- or Nanodrop-type measurements, requiring the adaptation of the standard A280 protocols for use in 96-well microplates. While many of our findings about how to achieve this adaptation successfully are available in the literature if one knows where to look, we have summarised the requirements in Supplementary Note 1, in the hope these will be useful to readers unfamiliar with these sources (see also Supplementary Fig. 4-8). In brief, the best results for A280 assays are obtained by using UV-clear plastic, removing additives such as imidazole, correcting for path length variation, and correcting for light scatter via analytical methods. This requires the collection of an absorbance spectrum from 200-1000 nm rather than just one reading at 280 nm, and is processed by two consecutive R functions, plot_absorbance_spectrum() and get_conc_a280() (Supplementary Fig. 6-7).
Systematic testing of the calibration protocol allows method validation

We sought to conduct a systematic assessment of the BCA and A280 methods by testing three spectrally distinct FPs in two buffers, assessed with both assays in parallel (Fig. 3). The chosen FPs (mTagBFP2, mGFPmut3 and mCherry) are widely used, monomeric, reasonably fast-maturing and bright. All three are almost identical on the protein level to their FPbase entries (Fig. 2B; Materials & Methods; Supplementary Data), with the exception that they all have a His<sub>6</sub> tag N-terminal extension, and mGFPmut3 includes a well-defined monomeric A206K mutation (Zacharias et al., 2002; Shaner et al., 2005). The chosen buffers (T5N15 (5mM Tris-HCl pH 7.5, 15 mM NaCl) and T5N15 with protease inhibitors) were both compatible with the microBCA assay (Supplementary Fig. 2B), however, pilot studies suggested they might have different effects on the A280 assay. Following purification, FPs were initially dialysed to remove additives, then re-dialysed into the assay buffer (Fig. 3A). Each FP:buffer combination was then serially diluted, and subjected to an absorbance scan (200-1000 nm measurement, for the A280), a fluorescence assay (fluorescence measurement with appropriate filters for each FP) and a microBCA assay (reagent addition, incubation and A562 nm measurement). The results of this comparative test are shown in Fig. 3B-C, Supplementary Fig. 9 and Supplementary Tables 1-2. Broadly, the results from each assay validate those of the other assay: the measured concentration of each FP using the microBCA and A280 assays are within 2-fold of each other for most samples (Fig. 3C) and apparent linear ranges reach ~1 ng/µl for each assay (Fig. 3B), an impressive result for the A280 assay considering that the reported sensitivity on the Nanodrop is ~100-fold higher (Thermo Fisher Scientific, 2010). However, it can be noted that there is some buffer sensitivity for both assays – the microBCA produces more linear results in the buffer containing protease inhibitors, whereas the A280 does better in the buffer without them.
Conversion factors obtained from different purification batches gave similar estimates where concentration estimates were made using optimal assay:buffer pairings, varying between 1.02 and 1.63-fold (Supplementary Fig. 9B).

**FPbase-enabled ECmax method performs better than conventional assays**

We trialled a third protein assay during this experiment, designated here as the ‘ECmax’ method. The principal idea behind this assay is that the A280 extinction coefficient, recorded in units of M$^{-1}$cm$^{-1}$, that relates a protein’s absorbance at 280 nm to its concentration, is not the only known extinction coefficient for FPs. FPs also possess an extinction coefficient (‘EC’) corresponding to their light absorption at their peak (‘max’) excitation wavelength. As the ‘ECmax’ of most FPs is available on FPbase, we can automate its retrieval using the FPbase API (Lambert, 2019). The analytical processing steps for the ECmax assay (in get_conc_ecmax()) are similar to those of the A280 assay (Supplementary Fig. 8), and require no further readings other than those completed for the A280 assay. As the maximal absorbance peaks for all FPs tested were higher than those at 280 nm (Supplementary Fig. 1B, Supplementary Table 3), we anticipated that the ECmax assay would be more sensitive. Further, as protease inhibitors absorb at wavelengths under 300 nm, we hypothesised this assay may be less buffer-sensitive than the A280. Compellingly, both appear to be true: we consistently found that the ECmax assay produced larger linear ranges and lower limits of detection than the other assays (approaching 0.1 ng/µl, 10-fold better than the A280 assay and 1000-fold better than a Nanodrop), and that it produced almost indistinguishable results whether or not the buffer contained protease inhibitors (Fig. 3B-C, Supplementary Fig. 9, Supplementary Table 1). In addition, predictions from the ECmax assay closely match those from the A280, typically predicting concentrations
matching at 80-100% those of the expected result (rather than 170-220% for microBCA; Fig. 3C, Supplementary Table 3). For these reasons, we propose that the ECmax assay would be the most robust assay to include in a simple calibration protocol and will proceed using concentrations calculated from ECmax assays in what follows.

Fluorescence assays are not linear in all buffers

Along with protein concentration, fluorescence assays were carried out for each FP to obtain relative fluorescence units to relate to FP molecules in each well. One unexpected observation from this experiment was that fluorescence assays produce textbook linear responses to concentration in buffers containing protease inhibitors, but without these, a steep fluorescence drop is observed, particularly below ~10^{12} molecules per well (Fig. 3D). On first encounter, this result can appear as though it were caused by pipetting error. However, for this experiment, microplates containing dilution series using both buffers were prepared in parallel by the same person, at the same time, using the same equipment, and the result was reproducible across different days and FPs. The lack of linearity is suspected to be due to FP destabilisation in T5N15 buffer that doesn’t contain any stabilising agents. In such case, we suspect protease inhibitors could act to provide stabilisation in the T5N15+pi buffer. Alternatively, it may be due to protein adsorption to the plastic at very low concentrations (Aitken and Learmonth, 2002), which can be successfully avoided by using a buffer containing protease inhibitors. In either case, the observed non-linearity results in an underestimation of the conversion factor in these buffers that ‘scales’ with gain and may result in an underestimation of conversion factor values of up to 8-fold (Fig. 3E), suggesting that the removal of protease inhibitors is inadvisable for an accurate calibration.
ECmax assay enables FP calibration without protein purification

Having established that the ECmax assay, a protein quantification assay that relies only on the peak light absorbance of each FP, is highly accurate and sensitive for purified proteins measured using trusted methods (Fig. 3), we asked whether this method could enable us to drop the purification step altogether. Dropping the purification step is not possible for the other assays as they are designed to quantify any and all proteins, but the ECmax should in principle be specific for the considered FP and may therefore be used to quantify FPs in crude lysates. This requires that cell lysates, complex mixtures of all the soluble proteins from a lysed cell, do not significantly absorb at the relevant wavelengths. To investigate this, we harvested and lysed cells expressing our three FPs, separated the soluble fraction, and concentrated it (~16-fold). Putting these through an ECmax assay and fluorescence assay, we observed almost identical conversion factor values as from our purified proteins (Supplementary Fig. 10). Of two lysis methods – sonication and use of a commercial lysis reagent – the former produces more accurate results. It therefore appears that by establishing the ECmax assay for FP quantification, it is possible to remove the purification step altogether without compromising calibration accuracy.

Calibration allows comparison across instruments for FPs other than GFP

While fluorescein enables the comparison of experimental results across laboratories and instruments by converting arbitrary units into ‘molecules of equivalent fluorescein’ (MEFL) units, FP calibration in principle offers the same capability by converting arbitrary units into units of ‘molecules of equivalent FP’ (MEFP), which is carried out using our R package’s process_plate() function (Fig. 4A-B). This allows us to quantify the number of FP molecules in each well of our microplates. To calculate uncalibrated ‘per cell’ values, typical studies...
will divide the RFU values by the optical density (OD600 or OD700) of the culture, which quantifies cell density. The calibration of optical density to particle number can be achieved through a similar calibration process using microspheres of similar size to *E. coli* (Stevenson *et al.*, 2016; Beal *et al.*, 2020). Using both calibrations, it is possible to quantify molecule number per cell in ‘molecules of equivalent FP per particles of equivalent microspheres’ (MEFP/PEMS, Fig. 4C), units which should allow cross comparison between different instruments, gains and filter sets. To test this, overnight cultures of *E. coli* containing mCherry expression vectors were split into separate but identical microplates containing arabinose, and were grown in two plate readers using a range of settings. The results show that normalised values of relative fluorescence differ by ~1.5, ~3 and ~130-fold without calibration, whereas such values become reliably comparable after calibration, even for experiments conducted using instrument settings that produce values that cannot be legibly plotted on the same axis (Fig. 4D).

**Calibration allows estimation of absolute cellular protein concentration**

We next asked if calibration to units of MEFP/PEMS is a reasonable approximation for molecule number per cell (Fig. 5A). We carried out microsphere calibration using 1 cm cuvettes and a standard spectrophotometer and obtained conversion factors (Supplementary Table 4) that fell within the range quantified by empirical OD600-specific cell counts (Volkmer and Heinemann, 2011). In addition, other authors have confirmed that values of fluorescent protein per cell using fluorescein and microsphere calibrants (MEFL/PEMS) are approximately equal to those obtained using fluorescein-calibrated single cell data on a flow cytometer (Beal *et al.*, 2020; Fedorec *et al.*, 2020), suggesting particle counts are likely to approximate actual cell numbers. However, we observed a major caveat
to the use of microspheres as calibrants, which is that their absorbance profiles differ from that of cells. This can frustrate their ability to provide accurate conversions between ‘per cell’ data calculated using OD700 versus OD600 measurements (Supplementary Fig. 11 and Supplementary Tables 4-5) for which we discuss solutions in Supplementary Note 2.

The question of whether the measured fluorescence of FPs in cells is the equivalent of measured fluorescence of the same number of FPs in vitro is less clear. Some authors have found that cells attenuate (or ‘quench’) fluorescence (Zhang et al., 2009; Hirst et al., 2015), but the magnitude of the effect has not been systematically investigated, particularly for modest cell concentrations found in a typical E. coli growth assay. We quantified the quenching properties of E. coli cells on our three FPs by mixing an increasing concentration of non-fluorescent cells with purified FPs, and quantifying the difference in apparent fluorescence with added cells (Fig. 5B and Supplementary Fig. 12). Our results suggest that this ‘quenching’ effect amounts to less than 20% of the fluorescence signal for moderate cell densities (OD600/cm under 0.5), but increases to ~30% for the highest cell densities typically observed in microplate-scale cultures (OD600/cm around 2). This information was used to add a correction step into the process_plate() calculations so as to compensate for the expected percentage loss of fluorescence with increasing cell density (Fig. 5B, right panel). The complete analytical workflow from calibration to experimental data processing is illustrated in Supplementary Fig. 13.

Using these amendments, we can show that it is possible to convert response curve assay data into molecules per cell. Figure 5C shows one experiment using mCherry expression construct in two vectors with different origins of replication. Using these vectors, we obtain...
figures for mCherry abundance that vary between \(~3,000\) to \(80,000\) molecules per cell for p15A, but \(~1000\) to \(600,000\) molecules per cell for colE1. Protein abundance information, available from proteomics and ribosome profiling studies, suggests that the typical \(E.\ coli\) protein is present in the order of 100s-1000s copies per cell, and the most abundant are present in the order of \(10^5\) copies per cell or higher (Ishihama et al., 2008; Arike et al., 2012; Li et al., 2014). Over-induction using the high-copy (colE1) vector therefore appears to allow synthetic protein expression to reach the level of the most abundant proteins in the cell. This is supported by the fact that these vectors reliably overexpress FPs to a level observable by SDS-PAGE in unpurified lysates (Fig. 1, Supplementary Fig. 1). Modest expression (in the order of \(10^2\)-\(10^3\) per cell) can be achieved by combining low arabinose concentrations with either vector. Leaky expression can be quantified in the colE1 vector as contributing hundreds of mCherry molecules per cell even without induction, whereas in the p15A vector it was merely ten copies per cell. In other words, using an average colE1 vector allows us to utilise the full spectrum of protein abundances from modestly expressed enzymes such as the RecBCD helicase (~100 copies per cell) through to the most abundant ribosomal proteins (~100,000 copies per cell, Li et al., 2014). We can also use this to compare the number of molecules produced from the same vectors but two different FPs. Interestingly, measurements from identical SEVA vectors revealed that while the FP abundances are in the same order of magnitude, mTagBFP2 accumulated to higher levels per cell than mCherry by 1.83-fold on average, despite sharing the same promoter, 5’ untranslated region, ribosome binding site and N-terminal protein sequences (Fig. 5C-D). Larger discrepancies were observed with colE1 which induced a relatively high leak in expression observable at low arabinose levels, and resulted in higher mCherry accumulation than mTagBFP2 at maximum induction.
In 2011, Volkmer and colleagues noted that while OD-specific *E. coli* cell counts varied with growth rate, the OD-specific total cell volume was approximately 3.6 µl per OD600/cm, regardless of strain or growth condition. Using OD as a measure of the cumulative cellular volume in a culture can therefore be used to convert fluorescence and OD measurements into concentrations in molar units, instead of ‘per cell’ values, and such conversions may be more appropriate for comparing experimental results with quantitative modelling of cellular reaction networks, since it is unaffected by growth rate differences. Using this method, we find that FP abundances using the same vectors populate a range of concentrations between 0.01-100 µM (Fig. 5D).

Absolute quantification reveals hidden properties of fluorescent proteins

Finally, we were interested in testing whether the quantification of fluorescent proteins in cells could shed more light on the question of how to avoid quantification errors in a typical experiment. The presence of red FPs has been suggested to interfere with bacterial cell density estimations at 600 nm since red FPs typically absorb well at this wavelength (Hecht *et al.*, 2016) and has led to the conclusion that circuits using RFP must be quantified at 700 nm, which is unaffected by their presence. However, the number of molecules of red FPs that might be required for this effect to occur has never been quantified.

Calibrated timecourse data of mCherry overexpression in *E. coli* (Fig. 6) was examined to quantify these effects, with mGFPmut3 and mTagBFP2 used as negative controls. The ratio between OD600 and OD700 measurements was used to identify errors caused by red FP absorbance. Linear models fitted to the relationship between measured OD600 and OD700...
values confirmed that this relationship was very similar for all uninduced cells (Fig. 6A; OD600 = 1.30 * OD700 – 0.02), but mCherry induction resulted in a measurable deviation (OD600 = 1.37 * OD700 – 0.03, Fig. 6A-C): similar to what Hecht and colleagues measured (Hecht et al., 2016). However, the relationship between this shift and cellular protein copy number was not previously investigated. Looking at this relationship (Fig. 6D), our results indicate that the error due to measuring cell density using OD600 values for mCherry is only apparent when mCherry levels per cell are high (over 100,000 per cell), and that the magnitude of this difference reaches only ~5 %. In other words, the effect is smaller in magnitude than errors from cell quenching, and only affects systems where mCherry is overexpressed. In contrast, mGFPmut3 expression to similar levels has no effect on cell density estimation using OD600, as expected. Surprisingly, we observed the opposite trend for mTagBFP2, in which, assuming OD700 produces a correct estimate, OD600 appears to underestimate cell density where mTagBFP2 is expressed at high levels per cell. The reasons for this are currently unclear and beyond the scope of this paper, but our results suggest that this could be an interesting avenue for future work. Generally, this experiment confirmed that the presence of low to moderate FP levels per cell (under 100,000) do not perturb cell density estimates, and errors are of a lower magnitude in all cases than those from cellular fluorescence quenching.

Discussion

Our aim for this work was to develop a generalisable method that allows fluorescence readings on microplate readers to be calibrated to molecular units of fluorescent protein. The method ought to be (1) accurate and sensitive, (2) as simple as possible, (3) suitable for
any fluorescent protein, and (4) easily analysed. To develop the method, we adopted the principles of redundant experimental design, including the validation of multiple assay types, characterisation of the method’s consistency, and the assessment of its generality for three different FPs (Casadevall and Fang, 2016).

Our initial method using a simple purification protocol (Fig. 2) and a commercial protein assay allowed us to develop an analysis pipeline to obtain conversion factors from purified FP calibrants. To demonstrate the accuracy and validity of our calibrations, we verified that the absorbance and fluorescence spectra of the calibrants matched their counterparts on FPbase (Fig. 1, Supplementary Fig. 2; Raynal et al., 2014; Lambert, 2019), and validated our initial protein assay measurements by cross comparison with two further methods. To do this, we adapted the low-throughput A280 assay into an accurate, high-throughput assay format, and showed that these were suitable for use with FPs even though some absorb in the near-UV range. This type of assay for FP quantification has not, to our knowledge, been demonstrated in the literature before, and we contend that it is likely to be of particular interest since it requires no calibrant or commercial reagent, no expensive quartz-based consumables and exhibits a sensitivity that exceeds that of commercial systems such as the Nanodrop.

We also discovered a methodological ‘hack’ to obtaining FP concentrations using the extinction coefficients at their maximum excitation wavelength, the ECmax assay, which was both the simplest and the most robust of all the assays tested. Specifically, the ECmax assay was the least affected by buffer conditions, and had the largest linear range (to ~ 0.1 ng/µl; Fig. 3). We note that the assay is limited by the fact that it requires the used FP to be
documented on FPbase, and assumes that the documented ECmax measured by other
laboratories is accurate. Promisingly, our results suggest good inter-lab agreement for these
measurements (compare A280 and ECmax estimates). In addition, an analysis of all FPs on
FPbase, comparing the proteins’ extinction coefficients at 280 nm versus at their maximum
excitation wavelength, supports the idea that the ECmax is a more sensitive assay for most
FPs (Supplementary Fig. 14, Supplementary Table 3). While the EC(280) values are highly
uniform (median: ~27,400 M\(^{-1}\)cm\(^{-1}\)), likely because most FPs are very similar in size, the
ECmax values are mostly considerably larger (median ~64,200 M\(^{-1}\)cm\(^{-1}\)). Impressively, as the
ECmax assay specifically quantifies FP concentration rather than total protein concentration,
we were also able to show that it is possible to do these calibrations in crude lysate without
compromising on accuracy, demonstrating that calibrants may be produced without affinity
purification (Supplementary Fig. 10). Overall, we expect calibrations carried out using the
ECmax method, using either purified calibrants or (sonicated) cell lysates, to be equally
accurate. (Alternatively, if necessary, users can purify FPs and cross-check quantifications
using multiple assays.) We provide both rigorous and expedient protocols for prospective
users on protocols.io.

Such calibrations can then be used, first, to enable the comparison of experimental results
from different plate readers or across different settings, in molecules of fluorescent protein
per particles of equivalent microspheres (MEFP/PEMS; Fig. 4B). This is akin to using
fluorescein but with a broader application range, since using bespoke calibrants for each FP
allows us to calibrate instruments for any FP regardless of its spectral characteristics. If used
merely as comparative units, the precision (repeatability) of each calibration is important,
but their accuracy (whether conversions predict molecule numbers as closely as possible) is not.

Second, they can be used to express protein abundance as ‘molecules per cell’ (Fig. 5C). Accuracy here is an important consideration, and will not only depend on the accuracy of the FP calibration (discussed above), but also on the microsphere calibration, and the removal of any interactions between absorbance and fluorescence characteristics of cells expressing FPs. As to microsphere calibration, cross-comparison with flow cytometry data suggests cell count estimates from microsphere calibrations are reasonable (Beal et al., 2020; Fedorec et al., 2020), although Beal and colleagues used 0.961 µm microspheres whose size is closer to *E. coli* than those used in this study (0.890 µm; the larger type are now unavailable). Our protein abundance estimates (10^2-10^5 proteins per cell; Fig. 5B) are also within reasonable bounds (Ishihama et al., 2008; Li et al., 2014), corroborating their use and indicating that FP calibrations may enable protein abundance comparisons between microplate assays and proteomics experiments.

The consideration of whether the presence of cells interferes with fluorescence quantification (or vice versa) is multifaceted. It is well known that cells interfere with fluorescence measurements through autofluorescence. Cellular autofluorescence is known to largely impact GFP quantification accuracy (Lichten et al., 2014), and is corrected for in FPCountR by normalising to the background fluorescence of control cells at a similar OD (akin to Hirst et al., 2015; Fedorec et al., 2020). The ‘quenching’ of apparent FP fluorescence by the presence of cells is more rarely considered (Hirst et al., 2015). We have found that the effect size is comparable for different FPs (Supplementary Fig. 12), unlike for
autofluorescence, supporting previous observations by others (Zhang et al., 2009). A correction for this attenuation in FPCountR compensates fluorescence according to the expected percentage quenched at the measured OD (Fig. 5B). Both of these corrections are included in the process_plate() function.

Certain sources of error cannot be adequately addressed by calibration alone. While some authors note that pH can affect the molecular brightness of certain FPs (Hirst et al., 2015), it could not be compensated for analytically without user input detailing both the pH response profile of the included FPs and the pH of their cells. Fortunately, since the cellular pH in *E. coli* is limited between pH 7.2-7.8 (Wilks and Slonczewski, 2007), and even pH-sensitive FPs exhibit only mild (<10%) variation in molecular brightness between pH 7-8 (Kneen et al., 1998; Roberts et al., 2016), pH-dependent changes in molecular brightness are unlikely to have a large effect on quantifications.

Overall, it seems likely that the accuracy of calibrated molecule counts per cell may be limited by the accuracy of cell count calibration. It is known that OD-specific cell counts change with growth rate, and that this is due to the positive relationship between growth rate and cell size for *E. coli* (Volkmer and Heinemann, 2011). We therefore believe these calculations of protein counts per cell will be more accurate where cell size and microsphere size are as similar as possible, and inaccuracies will arise if the differences become significant. Thus, these values are likely to be approximately accurate so long as the following assumptions are true: that *E. coli* absorbance (scatter) is well represented by microsphere properties, and that it doesn’t change significantly over time or between samples. These assumptions are likely to be true for typical *E. coli* experiments since cell
shrinkage has been shown to take place only after several hours in stationary culture, and only large growth rate differences maintained for prolonged periods were observed to make a significant difference to cell size in exponentially growing cells (Volkmer and Heinemann, 2011). In addition, our finding that microsphere absorbance profiles differ from *E. coli* ones suggest the use of caution when comparing between OD600 and OD700 measurements calibrated using microspheres (Supplementary Note 2 and Supplementary Fig. 11). In contrast, using a conversion from OD to total cell volume in a given culture well allows us to remove the requirement for counting cells (Fig. 5D) and allows the expression of protein concentrations as ~0.01-100 µM. These figures should be approximately accurate under the assumption that the OD-specific cell volume doesn’t vary significantly between samples or over time. This is strongly supported by the results of Volkmer and colleagues (Volkmer and Heinemann, 2011) whose data suggests this variation is within ~2-fold across a wide range of growth conditions, but also by others who have shown that as cell volumes increase, their OD-specific cell counts decrease by approximately the same magnitude (Schaechter *et al.*, 1958; Basan *et al.*, 2015). Units of concentration may also be more meaningful for reaction modelling since ultimately it is molecular concentration that is critical for binding and kinetics (Bloom *et al.*, 2014; Siegal-Gaskins *et al.*, 2014; Boada *et al.*, 2019).

Absolute FP quantification should allow us to make systematic comparisons between different FPs in synthetic circuits. While such a comparison was beyond the scope of this paper, we did observe that while our standardised SEVA vectors produced mCherry and mTagBFP2 on a comparable order of magnitude, there were observable differences between the levels of each FP. This supports the idea that the use of identical transcriptional and translational elements cannot always be assumed to guarantee
equivalent translation (or degradation) rates of different fluorescent proteins (Tuller et al., 2010; Tuller and Zur, 2015; Cambray et al., 2018), and is not a good assumption on which to base FP calibrations, as suggested elsewhere (Vignoni et al., 2019).

We used absolute protein quantification to investigate the problem first described by Hecht and colleagues (Hecht et al., 2016) in which an association between mCherry overexpression and deviations in cell density measurements were made. Repeating these assays showed a clear effect, but this effect was of modest magnitude (<5%) and was only apparent at very high mCherry levels per cell (over $10^5$ molecules per cell; Fig. 6). This suggests that for most circuits that use moderate expression levels to minimise cellular burden, OD600 values would remain an accurate way to quantify cell density. We also unexpectedly observed the opposite effect in mTagBFP2 expressions. As this analysis was technically a quantification of whether OD600 and OD700 measurements deviate from each other in the presence of different FPs, these results might not suggest that OD600-based cell density readings in the presence of high mTagBFP2 are inaccurate. It might instead suggest that, conversely, for mTagBFP2, the OD700 readings are inaccurate. We do not currently have an explanation for this finding. One possible contributing factor may be that while it is often described as one of the best blue FPs, mTagBFP2 has a higher propensity to aggregate than mCherry and most GFPs (Cranfill et al., 2016). Cell stress is known to induce *E. coli* to elongate (Justice et al., 2008; Basan et al., 2015), which may affect its scattering properties. It is possible that very high FP levels may frequently cause a small but significant error in cell density estimates due to combinations of effects from light absorption and scatter that warrants more study in order to allow us to further improve molecular quantifications of FPs under those conditions.
Absolute quantification need not be limited to fluorescent proteins. The last few years have seen a fantastic expansion of fluorogenic molecules, tools that have allowed the specific quantification of localised proteins (Svendsen et al., 2008; Li et al., 2018; Csibra et al., 2020), proteins in anaerobic environments (Streett et al., 2019; Charubin et al., 2020), and the fluorescent quantification of RNAs (Pothoulakis et al., 2014; Siegal-Gaskins et al., 2014; Yerramilli and Kim, 2018). Calibration of these molecules would be more complex to achieve but no less valuable. Calibration of alternative instruments such as flow cytometers are also of interest, but would require a very different approach, requiring calibrants to be attached to particles of cell-like sizes.

While flow cytometry and mass spectrometry allow us to probe single-cell measurements or the protein complement of an entire cell, respectively, microplate readers remain an important screening platform in the Design-Build-Test-Learn cycle due to ease of use, low cost and high iterative capabilities. This necessitates the development of methods for extracting informative numbers from such data. We hope that our demonstration of how to achieve absolute FP quantifications using FPCountR can contribute to the effort to develop more quantitative approaches for the analysis of circuit behaviour in synthetic biology.

**Materials and Methods**

**Materials**

Primers and gblocks were obtained from IDT, and *E. coli* strain DH5-alpha (Invitrogen, 18265-017) was used for molecular cloning. Chemicals and protein reagents were purchased
Fluorescent proteins

The mCherry (Shaner et al., 2004) protein sequence was based on the FPbase entry for mcherry (FPbase ID: ZERB6) with the following changes: M1(MVHHHHHHGSG). The mGFPmut3 (Cormack, Valdivia and Falkow, 1996) protein sequence was based on the FPbase entry for gfpmut3 (FPbase ID: A20WC) with the following changes: M1(MVHHHHHHGSG), A206K (a substitution to make the protein monomeric; (Zacharias et al., 2002; Shaner, Steinbach and Tsien, 2005)). The mTagBFP2 (Subach et al., 2011) protein sequence was based on the FPbase entry for mtagfp2 (FPbase ID: ZO7NN) with the following changes: M1(MVHHHHHHGSG). Full protein sequences are provided in Supplementary Table 6.

DNA assembly

Vectors for fluorescent protein purification and growth curve assays were constructed according to standard protocols, via Golden Gate and Gibson assembly techniques using E. coli strain DH5α (Invitrogen, 18265-017). Constructs were assembled into Standardised European Vector Architecture (SEVA) backbones (Silva-Rocha et al., 2013): pS381 was generated from pS181 with chloramphenicol substitution; pS361 was generated similarly from pS161. In an effort to approximately equalise expression levels between different proteins, the 5’ untranslated region (including ribosome binding site) and 5’ region of each construct was set to be identical up to residue 11 (coding region begins: DNA:..
ATGGTTCACCATCATCACacGGTtcgggc, protein: MVHHHHHHHGSG). The second residue was set to valine to reduce the effects of N-end mediated degradation (Mogk, Schmidt and Bukau, 2007). The affinity tag chosen for purification was the His\textsubscript{6} tag, which was followed by a short unstructured linker (GSG). Full DNA sequences of vectors are provided in Supplementary Table 6.

**FPCount (wet lab) protocol**

The protocols are available on protocols.io. In total, we have described three FPCount calibration protocols: (1) the full protocol using FP purification and three protein assays for cross-validation, as conducted in Fig. 3 available at https://www.protocols.io/view/fpcount-protocol-full-protocol-bztsp6ne (Csibra and Stan, 2021a), (2) a short protocol requiring purification but using the ECmax assay only, available at https://www.protocols.io/view/fpcount-protocol-short-protocol-bzt6p6re (Csibra and Stan, 2021c), and (3) the purification-free protocol that uses FPs in lysates for calibration, available at https://www.protocols.io/view/fpcount-protocol-in-lysate-purification-free-proto-bzudp6s6 (Csibra and Stan, 2021b). It is anticipated that users will have differing requirements concerning protocol time and validation, so we have provided all three protocols.

**Protein expression and harvesting**

Fluorescent proteins were produced using pS381 (SEVA) expression vectors in *E. coli* BL21(DE3) strains. Glycerol stocks were inoculated into 50 ml Luria Broth (Miller) supplemented with 50 µg/ml chloramphenicol and 0.02 % arabinose and were grown overnight at 30 °C at 250 rpm. Cells were harvested after ~16 h by transferring them to
prechilled containers on ice. All further steps were conducted on ice. OD600 readings were taken, and 40 OD of cells was transferred to fresh falcon tubes, washed once in T50N300 (50 mM Tris-HCl pH 7.5, 300 mM NaCl) and resuspended in lysis buffer (T50N300, 1X protease inhibitors (EDTA-free, Pierce A32955), filter sterilised, supplemented with lysozyme 100 µg/µl). Cells were separated into 20 OD (2 ml) fractions and sonicated (Qsonica Q125 sonicator, 50% amplitude, 10s on, 10s off, 2 min). Lysates were supplemented with 5 mM CaCl$_2$, 50 mM MgCl$_2$ and treated with DNase I (50 U/ml, bovine pancreas, MP Biomedicals, 219006210) for 30 min at 4 °C. Soluble fractions were isolated by isolation of the supernatant after centrifugation (16,000 xg, 30 min, 4 °C), and both fractions were checked by SDS-PAGE followed by staining with Coomassie-based dye according to the manufacturer’s instructions (Instant Blue Protein Stain, Sigma ISB1L-1L).

**Protein purification**

Fluorescent proteins were purified in batch using His-tag affinity chromatography at room temperature according to the resin manufacturer’s instructions (Thermo Fisher). Lysates were supplemented with 10 mM imidazole and 600 µl was applied to HisPur Cobalt resin (300 µl, ThermoFisher) equilibrated in Binding Buffer (T50N300+pi, 10 mM imidazole), mixed and incubated at room temperature for ~15 min before removal (1,000 xg, 1 min). This was repeated four times, before the resin was washed with 10 column volumes of Binding Buffer. Protein was eluted in Elution Buffer (T50N300+pi, 150 mM imidazole). All protein fractions for calibration were stored protected from light at 4 °C.
Preparation for calibration assays

Elution fractions were combined and concentrated approximately 10-fold using Amicon centrifugal filter columns (Merck, UFC5010), followed by buffer exchange (1000x) into T5N15 (5mM Tris-HCl pH 7.5, 15 mM NaCl) or T5N15+pi (T5N15, 1x protease inhibitors, filter sterilised).

Microplate reader assays. All assays were carried out using a Tecan Spark microplate reader except the fluorescence spectra assays, which were carried out using a BMG Clariostar Plus microplate reader.

Calibration assays. For each FP calibration, both concentration and fluorescence assays were carried out on the same dilution series of protein. Concentrated, buffer exchanged FP (100 µl) was diluted in 900 µl buffer, then diluted 1:2 into 500 µl buffer in 1.5 ml eppendorfs. A total of 11 dilutions were prepared in this way, distributed into UV-transparent microplates (Greiner, 655801) as duplicates (225 µl). Bovine serum albumin (BSA) standards (from Micro BCA Protein Assay kit, ThermoFisher, 23235) were prepared in parallel with the same buffer(s). This dilution set was then subjected to the protein concentration and fluorescence assays.

Protein concentration assays: A280 assay and ECmax assay

Absorbance assays were carried out on 225 µl protein in UV-transparent plates, using the Spark absorbance scan method (see Supplementary Methods).
Fluorescence assays

Following absorbance scans, 200 µl from each well of the original plate was transferred into clear polystyrene plates (Corning, 3370). This plate was sealed (Eppendorf Masterclear real-time PCR film adhesive, 30132947) and used to run the Spark fluorescence methods (see Supplementary Methods) on all relevant instruments, channels and gains.

Protein concentration assays: microBCA assay

BCA assays were carried out using the Micro BCA Protein Assay kit (ThermoFisher, 23235) according to the manufacturer’s instructions (microplate protocol). Briefly, 150 µl of working reagent was dispensed into a clean microplate, and 150 µl from each well of the fluorescence assay plate was mixed into the reagent with a multichannel pipettor. Reactions were covered with a plate seal (BreatheEasy sealing membrane, Sigma, Z380059), and subjected to the Spark microBCA method (see Supplementary Methods).

Calibration of OD600 and OD700 values using microspheres

Calibration of optical density readings used to quantify cell number (OD600 and OD700) was carried out according to published protocols (Fedorec et al., 2020). The microspheres used were monodisperse silica microspheres (Cospheric, SiO2MS-2.0, 2.0g/cc, d50=0.890um, CV=3.2%, <1% Doubles).

Bacterial timecourse assays

DH10B E. coli transformants were grown overnight in M9 medium (M9 salts (1X, Sigma M6030), casamino acids (0.2%), fructose (0.8%), thiamine HCl (0.25 mg/ml), MgSO₄ (2 mM), CaCl₂ (0.1 mM)) supplemented with 50 µg/ml chloramphenicol, in a deep-well plate (30 °C,
700 rpm), and diluted the following morning into fresh M9 with antibiotic (deep-well plate, 30 °C, 700 rpm) to an OD600 (cm⁻¹) of 0.05. After 1 hour, cultures were transferred into clear 96-well microplates (Corning, 3370) with pre-loaded arabinose (5 µl). Plates were sealed (BreatheEasy sealing membrane, Sigma, Z380059) and grown in a Tecan Spark plate reader in kinetic mode (see Supplementary Methods, Spark growth curve method).

42 Analytical methods

All data was analysed using R (R Core Team, 2021). The FPCountR package that was developed for the FP calibrations is available on GitHub at https://github.com/ec363/fpcountr (Csibra, 2021). For a summary of the functions, see the Fig. 1 and Supplementary Fig. 13.

48 Fluorescence scans

Excitation and emission spectra of the fluorescent proteins were conducted using a BMG Clariostar Plus microplate reader in sealed plates (Corning, 3370; Eppendorf Masterclear real-time PCR film adhesive, 30132947) at wavelengths appropriate to each FP (see Supplementary Methods).

44 Figures

Figures were created using RStudio and Biorender.com.

57 Data availability

Computer code produced in this study is available on the following repositories:

- R package for data analysis: GitHub (https://github.com/ec363/fpcountr)
This study includes no data deposited in external repositories.

Acknowledgements

GBS and EC acknowledge funding from the Royal Academy of Engineering (RAEng CIET 1819\5).

Author contributions

EC and GBS conceived the study. EC conducted the experiments, analysed the data and wrote the R package. EC and GBS wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

References

Aitken, A. and Learmonth, M.P. (2002) ‘Protein Determination by UV Absorption’, in Walker, J. M., Protein Protocols Handbook, The. New Jersey: Humana Press, pp. 3–6. doi:10.1385/1-59259-169-8:3.

Arike, L. et al. (2012) ‘Comparison and applications of label-free absolute proteome quantification methods on Escherichia coli’, Journal of Proteomics, 75(17), pp. 5437–5448. doi:10.1016/j.jprot.2012.06.020.

Basan, M. et al. (2015) ‘Inflating bacterial cells by increased protein synthesis’, Molecular Systems Biology, 11(10), p. 836. doi:10.15252/msb.20156178.

Beal, J. et al. (2018) ‘Quantification of bacterial fluorescence using independent calibrants’, PLOS ONE, 13(6), p. e0199432. doi:10.1371/journal.pone.0199432.

Beal, J., Farny, N.G., et al. (2020) ‘Robust estimation of bacterial cell count from optical density’, Communications Biology, 3(1), p. 512. doi:10.1038/s42003-020-01127-5.

Beal, J., Goñi-Moreno, A., et al. (2020) ‘The long journey towards standards for engineering biosystems: Are the Molecular Biology and the Biotech communities ready to standardise?’, EMBO reports, 21(5). doi:10.15252/embr.202050521.
Bloom, R.J., Winkler, S.M. and Smolke, C.D. (2014) ‘A quantitative framework for the forward design of synthetic miRNA circuits’, *Nature Methods*, 11(11), pp. 1147–1153. doi:10.1038/nmeth.3100.

Boada, Y. *et al.* (2019) ‘Characterization of Gene Circuit Parts Based on Multiobjective Optimization by Using Standard Calibrated Measurements’, *ChemBioChem*, 20(20), pp. 2653–2665. doi:10.1002/cbic.201900272.

Boo, A., Ellis, T. and Stan, G.-B. (2019) ‘Host-aware synthetic biology’, *Current Opinion in Systems Biology*, 14, pp. 66–72. doi:10.1016/j.coisb.2019.03.001.

Bradford, M.M. (1976) ‘A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding’, *Analytical Biochemistry*, 72(1), pp. 248–254. doi:10.1016/0003-2697(76)90527-3.

Calderón-Celis, F., Encinar, J.R. and Sanz-Medel, A. (2018) ‘Standardization approaches in absolute quantitative proteomics with mass spectrometry’, *Mass Spectrometry Reviews*, 37(6), pp. 715–737. doi:10.1002/mas.21542.

Cambray, G., Guimaraes, J.C. and Arkin, A.P. (2018) ‘Evaluation of 244,000 synthetic sequences reveals design principles to optimize translation in *Escherichia coli*,’ *Nature Biotechnology* [Preprint]. doi:10.1038/nbt.4238.

Casadevall, A. and Fang, F.C. (2016) ‘Rigorous Science: a How-To Guide’, *mBio*, 7(6). doi:10.1128/mBio.01902-16.

Ceroni, F. *et al.* (2015) ‘Quantifying cellular capacity identifies gene expression designs with reduced burden’, *Nature Methods*, 12(5), pp. 415–418. doi:10.1038/nmeth.3339.

Chalfie, M. *et al.* (1994) ‘Green fluorescent protein as a marker for gene expression’, *Science*, 263(5148), pp. 802–805. doi:10.1126/science.8303295.

Charubin, K., Streett, H. and Papoutsakis, E.T. (2020) ‘Development of Strong Anaerobic Fluorescent Reporters for *Clostridium acetobutylicum* and *Clostridium ljungdahlii* Using HaloTag and SNAP-tag Proteins’, *Applied and Environmental Microbiology*. Edited by R.M. Kelly, 86(20). doi:10.1128/AEM.01271-20.

Cormack, B.P., Valdivia, R.H. and Falkow, S. (1996) ‘FACS-optimized mutants of the green fluorescent protein (GFP),’ *Gene*, 173(1), pp. 33–38. doi:10.1016/0378-1119(95)00685-0.

Cranfill, P.J. *et al.* (2016) ‘Quantitative assessment of fluorescent proteins’, *Nature Methods*, 13(7), pp. 557–562. doi:10.1038/nmeth.3891.

Csibra, E. (2021) *FPCountR: Fluorescent protein calibration for plate readers*. Zenodo. doi:10.5281/zenodo.5760028.

Csibra, E., Renders, M. and Pinheiro, V. (2020) ‘Bacterial cell display as a robust and versatile platform for the engineering of low affinity ligands and enzymes’, *ChemBioChem*, p. cbic.202000203. doi:10.1002/cbic.202000203.
Csibra, E. and Stan, G.-B. (2021a) ‘FPCount protocol - Full protocol’, protocols.io [Preprint]. doi:10.17504/protocols.io.bztsp6ne.

Csibra, E. and Stan, G.-B. (2021b) ‘FPCount protocol - in-lysate (purification free) protocol’, protocols.io [Preprint]. doi:10.17504/protocols.io.bzudp6s6.

Csibra, E. and Stan, G.-B. (2021c) ‘FPCount protocol - Short protocol’, protocols.io [Preprint]. doi:10.17504/protocols.io.bzt6p6re.

Delvigne, F. et al. (2018) ‘Improving control in microbial cell factories: from single cell to large-scale bioproduction’, FEMS Microbiology Letters [Preprint]. doi:10.1093/femsle/fny236.

Fedorec, A.J.H. et al. (2020) ‘FlopR: An Open Source Software Package for Calibration and Normalization of Plate Reader and Flow Cytometry Data’, ACS Synthetic Biology, 9(9), pp. 2258–2266. doi:10.1021/acssynbio.0c00296.

Finan, K., Rauf, A. and Heilemann, M. (2015) ‘A Set of Homo-Oligomeric Standards Allows Accurate Protein Counting’, Angewandte Chemie International Edition, 54(41), pp. 12049–12052. doi:10.1002/anie.201505664.

Gorochowski, T.E. et al. (2019) ‘Absolute quantification of translational regulation and burden using combined sequencing approaches’, Molecular Systems Biology, 15(5), p. e8719. doi:10.15252/msb.20188719.

Hecht, A. et al. (2016) ‘When Wavelengths Collide: Bias in Cell Abundance Measurements Due to Expressed Fluorescent Proteins’, ACS Synthetic Biology, 5(9), pp. 1024–1027. doi:10.1021/acssynbio.6b00072.

Hirst, C.D. et al. (2015) ‘Protocol for the Standardisation of Transcriptional Measurements’, in McGenity, T.J., Timmis, K.N., and Nogales, B. (eds) Hydrocarbon and Lipid Microbiology Protocols. Berlin, Heidelberg: Springer Berlin Heidelberg (Springer Protocols Handbooks), pp. 9–26. doi:10.1007/8623_2015_148.

Ishihama, Y. et al. (2008) ‘Protein abundance profiling of the Escherichia coli cytosol’, BMC Genomics, 9(1), p. 102. doi:10.1186/1471-2164-9-102.

Justice, S.S. et al. (2008) ‘Morphological plasticity as a bacterial survival strategy’, Nature Reviews Microbiology, 6(2), pp. 162–168. doi:10.1038/nrmicro1820.

Kneen, M. et al. (1998) ‘Green Fluorescent Protein as a Noninvasive Intracellular pH Indicator’, Biophysical Journal, 74(3), pp. 1591–1599. doi:10.1016/S0006-3495(98)77870-1.

Lambert, T.J. (2019) ‘FPbase: a community-editable fluorescent protein database’, Nature Methods, 16(4), pp. 277–278. doi:10.1038/s41592-019-0352-8.

Li, C. et al. (2018) ‘Fluorogenic Probing of Membrane Protein Trafficking’, Bioconjugate Chemistry, 29(6), pp. 1823–1828. doi:10.1021/acs.bioconjchem.8b00180.
Li, G.-W. et al. (2014) ‘Quantifying Absolute Protein Synthesis Rates Reveals Principles Underlying Allocation of Cellular Resources’, Cell, 157(3), pp. 624–635. doi:10.1016/j.cell.2014.02.033.

Lichten, C.A. et al. (2014) ‘Unmixing of fluorescence spectra to resolve quantitative time-series measurements of gene expression in plate readers’, BMC Biotechnology, 14(1), p. 11. doi:10.1186/1472-6750-14-11.

Mogk, A., Schmidt, R. and Bukau, B. (2007) ‘The N-end rule pathway for regulated proteolysis: prokaryotic and eukaryotic strategies’, Trends in Cell Biology, 17(4), pp. 165–172. doi:10.1016/j.tcb.2007.02.001.

Noble, J.E. et al. (2007) ‘A Comparison of Protein Quantitation Assays for Biopharmaceutical Applications’, Molecular Biotechnology, 37(2), pp. 99–111. doi:10.1007/s12033-007-0038-9.

Noble, J.E. (2014) ‘Quantification of Protein Concentration Using UV Absorbance and Coomassie Dyes’, in Methods in Enzymology. Elsevier, pp. 17–26. doi:10.1016/B978-0-12-420070-8.00002-7.

Pace, C.N. et al. (1995) ‘How to measure and predict the molar absorption coefficient of a protein’, Protein Science, 4(11), pp. 2411–2423. doi:10.1002/pro.5560041120.

Perrino, G. et al. (2021) ‘Control engineering and synthetic biology: working in synergy for the analysis and control of microbial systems’, Current Opinion in Microbiology, 62, pp. 68–75. doi:10.1016/j.mib.2021.05.004.

Pothoulakis, G. et al. (2014) ‘The Spinach RNA Aptamer as a Characterization Tool for Synthetic Biology’, ACS Synthetic Biology, 3(3), pp. 182–187. doi:10.1021/sb400089c.

R Core Team (2021) R: The R Project for Statistical Computing. Available at: https://www.r-project.org/ (Accessed: 5 December 2021).

Raynal, B. et al. (2014) ‘Quality assessment and optimization of purified protein samples: why and how?’, Microbial Cell Factories, 13(1), p. 180. doi:10.1186/s12934-014-0180-6.

Roberts, T.M. et al. (2016) ‘Identification and Characterisation of a pH-stable GFP’, Scientific Reports, 6(1), p. 28166. doi:10.1038/srep28166.

Rodriguez, E.A. et al. (2017) ‘The Growing and Glowing Toolbox of Fluorescent and Photoactive Proteins’, Trends in Biochemical Sciences, 42(2), pp. 111–129. doi:10.1016/j.tibs.2016.09.010.

Schaechter, M., Maaløe, O. and Kjeldgaard, N.O.Y. 1958 (1958) ‘Dependency on Medium and Temperature of Cell Size and Chemical Composition during Balanced Growth of Salmonella typhimurium’, Microbiology, 19(3), pp. 592–606. doi:10.1099/00221287-19-3-592.

Shaner, N.C. et al. (2004) ‘Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein’, Nature Biotechnology, 22(12), pp. 1567–1572. doi:10.1038/nbt1037.
Shaner, N.C., Steinbach, P.A. and Tsien, R.Y. (2005) ‘A guide to choosing fluorescent proteins’, Nature Methods, 2(12), pp. 905–909. doi:10.1038/nmeth819.

Siegal-Gaskins, D. et al. (2014) ‘Gene Circuit Performance Characterization and Resource Usage in a Cell-Free “Breadboard”’, ACS Synthetic Biology, 3(6), pp. 416–425. doi:10.1021/sb400203p.

Silva-Rocha, R. et al. (2013) ‘The Standard European Vector Architecture (SEVA): a coherent platform for the analysis and deployment of complex prokaryotic phenotypes’, Nucleic Acids Research, 41(Database issue), pp. D666–D675. doi:10.1093/nar/gks1119.

Stevenson, K. et al. (2016) ‘General calibration of microbial growth in microplate readers’, Scientific Reports, 6(1), p. 38828. doi:10.1038/srep38828.

Streett, H.E., Kalis, K.M. and Papoutsakis, E.T. (2019) ‘A Strongly Fluorescing Anaerobic Reporter and Protein-Tagging System for Clostridium Organisms Based on the Fluorescence-Activating and Absorption-Shifting Tag Protein (FAST)’, Applied and Environmental Microbiology. Edited by M. Kivisaar, 85(14). doi:10.1128/AEM.00622-19.

Subach, O.M. et al. (2011) ‘An Enhanced Monomeric Blue Fluorescent Protein with the High Chemical Stability of the Chromophore’, PLOS ONE, 6(12), p. e28674. doi:10.1371/journal.pone.0028674.

Svendsen, S. et al. (2008) ‘Spatial separation and bidirectional trafficking of proteins using a multi-functional reporter’, BMC Cell Biology, 9(1), p. 17. doi:10.1186/1471-2121-9-17.

Tal, M., Silberstein, A. and Nusser, E. (1985) ‘Why does Coomassie Brilliant Blue R interact differently with different proteins? A partial answer.’, Journal of Biological Chemistry, 260(18), pp. 9976–9980. doi:10.1016/S0021-9258(17)39198-6.

Taniguchi, Y. et al. (2010) ‘Quantifying E. coli Proteome and Transcriptome with Single-Molecule Sensitivity in Single Cells’, 329, p. 8.

Thermo Fisher Scientific (2010) NanoDrop 1000 Spectrophotometer, V3.8 User’s Manual. Available at: https://tools.thermofisher.com/content/sfs/manuals/nd-1000-v3.8-users-manual-8%205x11.pdf (Accessed: 26 November 2021).

Thermo Fisher Scientific (2017) Protein assay technical handbook: Tools and reagents for improved quantitation of total or specific proteins. Available at: https://assets.thermofisher.com/TFS-Assets/LSG/brochures/protein-assay-technical-handook.pdf (Accessed: 26 November 2021).

Tie, H.C., Madugula, V. and Lu, L. (2016) ‘The development of a single molecule fluorescence standard and its application in estimating the stoichiometry of the nuclear pore complex’, Biochemical and Biophysical Research Communications, 478(4), pp. 1694–1699. doi:10.1016/j.bbrc.2016.09.005.

Tuller, T. et al. (2010) ‘An Evolutionarily Conserved Mechanism for Controlling the Efficiency of Protein Translation’, Cell, 141(2), pp. 344–354. doi:10.1016/j.cell.2010.03.031.
Tuller, T. and Zur, H. (2015) ‘Multiple roles of the coding sequence 5’ end in gene expression regulation’, *Nucleic Acids Research*, 43(1), pp. 13–28. doi:10.1093/nar/gku1313.

Vignoni, A. *et al.* (2019) ‘Fluorescence calibration and color equivalence for quantitative synthetic biology.** This work was partially supported by MINECO/AEI, EU DPI2017-82896-C2-1-R.’, *IFAC-PapersOnLine*, 52(26), pp. 129–134. doi:10.1016/j.ifacol.2019.12.247.

Volkmer, B. and Heinemann, M. (2011) ‘Condition-Dependent Cell Volume and Concentration of *Escherichia coli* to Facilitate Data Conversion for Systems Biology Modeling’, *PLoS ONE*. Edited by J. Langowski, 6(7), p. e23126. doi:10.1371/journal.pone.0023126.

Wasinger, V.C., Zeng, M. and Yau, Y. (2013) ‘Current Status and Advances in Quantitative Proteomic Mass Spectrometry’, *International Journal of Proteomics*, 2013, pp. 1–12. doi:10.1155/2013/180605.

Wilks, J.C. and Slonczewski, J.L. (2007) ‘pH of the Cytoplasm and Periplasm of *Escherichia coli*: Rapid Measurement by Green Fluorescent Protein Fluorimetry’, *Journal of Bacteriology*, 189(15), pp. 5601–5607. doi:10.1128/JB.00615-07.

Yerramilli, V.S. and Kim, K.H. (2018) ‘Labeling RNAs in Live Cells Using Malachite Green Aptamer Scaffolds as Fluorescent Probes’, *ACS Synthetic Biology*, 7(3), pp. 758–766. doi:10.1021/acssynbio.7b00237.

Zacharias, D.A. *et al.* (2002) ‘Partitioning of Lipid-Modified Monomeric GFPs into Membrane Microdomains of Live Cells’, *Science*, 296(5569), pp. 913–916. doi:10.1126/science.1068539.

Zhang, C. *et al.* (2009) ‘Correcting for the inner filter effect in measurements of fluorescent proteins in high-cell-density cultures’, *Analytical Biochemistry*, 390(2), pp. 197–202. doi:10.1016/j.ab.2009.04.029.
Figure 1. Overview of fluorescent protein calibration workflow using FPCountR. A calibration workflow is described (left), followed by a demonstration of how this calibration can be used to convert experimental data from arbitrary fluorescence units per optical density into molecules per cell (right). The calibration workflow consists of a wet lab protocol (top, available on protocols.io) and an analysis package (bottom, available on GitHub). In brief, the protocol describes how to prepare fluorescent protein calibrants by expression and purification, though the latter step is optional as lysates allow accurate calibration without the need for purification. The protocol also describes how to collect data for the calibration for both the protein assay to determine protein concentration, as well as the fluorescence assay to determine protein activity. The analytical workflow is provided as an open source R package, complete with functions that enable the extraction of protein concentrations from protein assay data, conversion factors (arbitrary fluorescence units per molecule) from a combination of protein and fluorescence assay data, and functions that allow users to convert experimental data into absolute units.
A high copy SEVA vector is expressed overnight. Lysis is performed by sonication, and the insoluble fraction is removed. His tag purifications are performed on a small scale using Cobalt resin, resulting in purified calibrants. These calibrants are used to prepare FP calibrants.

B The SEVA vector (pS381) is shown with FP calibrants. The table lists changes from FPbase:

| name         | FPbase slug       | changes from FPbase |
|--------------|-------------------|---------------------|
| mTagBFP2     | mTagBFP2:z2       | M1(MQHQQQHSGQG)    |
| mFPmut3      | gfpmut3:z2        | M1(MQHQQQHSGQG, A266K) |
| mCherry      | mCherry:z2        | M1(MQHQQQHSGQG)    |

C The mCherry calibrants are shown with bands at 58, 46, 32, and 25 kDa. M is the marker, S is soluble, I is insoluble, F is flowthrough, and E is elution.

D The mCherry calibrants are shown with bands at 58, 32, and 22 kDa, showing the FP calibrants.

E The wavelength distribution is shown for each calibrant, with peak values at different wavelengths.
Figure 2. Preparation of fluorescent protein calibrants.

A. Protocol summary. The use of high-copy vectors and overnight expression was designed to maximise protein production, and the temperature was dropped to 30°C to minimise misfolding. Cells were lysed using sonication, to avoid the requirement to add chemical components that may interfere with downstream processes, such as EDTA (with His-tag purification) or detergents (with protein quantification), or with unknown components of 'black box' commercial lysis reagents. Insoluble proteins were removed via centrifugation and SDS-PAGE was used to confirm that the majority of the expressed FP was in the soluble fraction. Proteins were purified using His-tag affinity purification. His tags are popular affinity tags that are known for their reliability and are small in size, making them unlikely to compromise fusion protein function. Cobalt resin was used as it has higher specificity than nickel resins and is expected to co-isolate fewer impurities. The arrows on the left represent the steps required to prepare purified calibrants (grey) vs. calibrants in lysate (black).

B. Vector and FP design. A standardised FP expression vector was constructed from an arabinose-inducible His-tagged FP construct in a high-copy SEVA vector. Three commonly-used FPs from across the spectral range were chosen for testing this protocol: mTagBFP2, mGFPmut3, and mCherry. A table of the three proteins illustrates any changes in protein sequence compared to their FPbase counterparts, showing identical matches with the exception of affinity tags and a monomerising mutation for GFPmut3.

C. Expression and solubility verification. SDS-PAGE analysis of lysates after separation of the insoluble fraction was used to make sure that most of the fluorescent protein was soluble. The displayed SDS-PAGE is from an mCherry purification, showing the separation of the soluble (S) vs. insoluble (I) fraction, next to the protein marker (M) on a 12% gel.

D. Purification verification. SDS-PAGE analysis after purification was used to confirm the success of purifications. The displayed SDS-PAGE is from an mCherry purification, showing the separation of the soluble (S) fraction, next to two flowthrough (F) fractions from the binding steps showing efficient FP binding to the cobalt resin, and three elution (E) fractions.

E. Fluorescence spectra. Fluorescence spectral scans were used to confirm that the purified FP behaves as expected. The figure shows obtained spectra (normalised such that the highest value = 1) fitted to a loess model with a 95% confidence interval (orange) overlaid with FPbase spectra (black) for each FP. Displayed spectra represent one sample measured in duplicate, that is representative of at least 2 independently purified batches of calibrant.
Figure 3. Protein assay comparison reveals that both assay linearity and limit of detection are sensitive to buffer components

**A. Sample preparation and assay workflow.** FPs in elution buffer (T50N300 with protease inhibitors (pi) and imidazole) were dialysed (1) into T5N15 and then again (2) into T5N15 or T5N15 with pi. Serial dilutions of each of these FPs in both buffers were then prepared, and each dilution was subjected to three protein assays (microBCA, A280 and ECmax assay) as well as the fluorescence assay. **B. Effect of buffer and assay on measured protein concentration of each dilution.** An example of how the buffer and choice of assay effect the measured protein concentrations using a dilution series of mTagBFP2 protein (for full results see Supplementary Fig. 9). The serial dilution was a 2-fold dilution, where 11 dilutions were prepared and measured in duplicate. Points represent the mean of the duplicate values. All dilutions were measured with the A280 and ECmax assays but only the top 8 dilutions were tested with the microBCA assay. The values for the A280 and ECmax assays were normalised for scatter as indicated in Supplementary Table 2. Any missing data points had concentrations recorded as being below 0.01 ng/µl. **C. Effect of assay on calculated protein concentration compared to the A280.** The raw data of each serial dilution, as displayed in B, was fitted to a linear model and used to estimate the concentration of the first sample in the series (where the dilution factor = 1), according to methods for each assay as set out in Supplementary Fig. 2, 7 and 8. For a given FP batch (set), the microBCA and ECmax assay error was calculated by taking the fold difference in concentration predicted by the named assay, versus that predicted by the A280 assay using T5N15 buffer. Each point therefore represents one value for each FP batch. The full data for this figure can be found in Supplementary Table 1. **D. Fluorescence assay sensitivity to buffer.** Combining the protein concentration predictions from the ECmax assay (x axis) with relative fluorescence units of the instrument to be calibrated from the fluorescence assay, conversion factors may be calculated by fitting a linear model of relative fluorescence units vs. molecules of protein. Points represent the mean of duplicate values at each concentration, and the line represents the fit found by the generate_cfs function. Patterns of linearity evident in these plots are representative of all fluorescence assays carried out in these two buffers. **E. Effect of buffer on conversion factor calculation.** Conversion factors were calculated for each protein, set and buffer using the modelled fits between relative fluorescence units and protein concentration from the ECmax assay, as in D. The fold difference in conversion factor prediction in buffers without (-) and with (+) protease inhibitors (pi) are displayed for mGFPM3, across both batches (set1 and set2) of protein. Each point therefore represents one value for each FP batch.
Figure 4. FP calibration allows comparison across gains and instruments for FPs other than GFP.

A. Calibration of fluorescence per well in units of MEFP. Timecourse experimental data of *E. coli* protein expression may be processed using the `process_plate()` function using conversion factors obtained from FP calibration. This allows the conversion of normalised data (in relative fluorescence units, rfu) to be converted into calibrated units, of ‘molecules of equivalent fluorescent protein’ or ‘MEFP’.

B. Comparison of normalised vs calibrated data in MEFP. Starter cultures of *E. coli* DH10B containing pS381_ara_mCherry were transferred into a 96-well plate. mCherry expression was uninduced (grey) or induced 0.1% (black) arabinose at 0 minutes. Absorbance at OD700 and fluorescence was monitored every ten minutes. Data was collected from three biological replicates, each of which is plotted. **Left panel:** normalised mCherry in units of RFU. **Right panel:** calibrated mCherry in units of MEFP. Inset plot shows the same data as the parent plot on a zoomed axis.

C. Calibration of fluorescence per cell in units of MEFP/PEMS. By combining the FP conversion factors with conversion factors from a microsphere calibration the data can be further processed using the `calc_fppercell()` function into ‘per cell’ data with units of MEFP per ‘particles of equivalent microspheres’ (PEMS).

D. Comparison of normalised vs calibrated data in MEFP/PEMS. Expressions were carried out as in B. Normalised and calibrated values are shown when compared across different filter sets (top, notation: emission wavelength/bandwidth), instruments (middle) and gains (bottom). Data for the filter and gain comparisons were taken using the same instrument.
Figure 5. Absolute quantification of *E. coli* timeseries data in molecules per cell.

**A. Functions to convert experimental data to absolute units.** Diagram of modifications to the `process_plate()` function to (i) incorporate a compensation step based on a quantitative understanding of the impact of cell density on apparent fluorescence (this allows the units to be recorded as molecules per cell), and (ii) to calculate molecular concentration of each FP instead, in molar units. **B. Quantification of the quenching effect on fluorescence on three FPs.** Purified FPs were mixed with non-fluorescent *E. coli* at a range of concentrations, and OD600 and fluorescence intensity were recorded. After normalising for cellular autofluorescence, the fold differences between relative fluorescence intensity (rfu) with (+) and without (-) added cells was quantified (left). Data was collected in duplicates, with both points plotted. A model was fit through this data to enable prediction of expected fluorescence quenching for a given cell density on experimental data. An example of the effect of the correction (right panel). An mCherry expression vector induced with low (open circles) and high (closed circles) concentration arabinose is presented without (grey) and with (black) correction. Data was collected from three biological replicates and all points are plotted. **C-D. Absolute protein quantification in molecules per cell (C) and molar concentration (D).** mCherry expression (top) from medium (pS361, p15A) and high (pS381, colE1) copy vectors was induced at a range of arabinose concentrations and quantified in a timecourse assay in a calibrated plate reader. Data was processed as described in (A) and cell estimates based on microsphere calibrations were used to calculate per cell values (C), or OD-specific cell volume data from Volkmer et al., 2011 was used to calculate molar concentrations (D). Data was collected from three biological replicates, each of which is plotted. (Bottom) mCherry expression from the top panel is compared with mTagBFP2 expression from an identical assay, plotted against arabinose concentration at the 420 min post induction. Data was collected from three biological replicates. Displayed points show the mean, and error bars indicate standard deviations.
Figure 6. Evaluation of OD600:OD700 ratios in cell growth assays.
Timecourse expression assays of three FPs conducted in a plate reader were monitored for OD600, OD700 and fluorescence intensity every 30 minutes, without (black) and with (red) arabinose. Data was collected from three biological replicates, and all points are plotted. This is a representative experiment of at least two independent experiments for each FP. 

A. Relationship between OD700 and OD600 values. Linear models were fitted to data from three FPs, and shown above each plot.

B. Timecourse assay data. Molecules of FP per cell calculated using calibrated FP and calibrated OD700 (B) and observed vs. expected OD600 values (C), plotted against time. Expected values were calculated from the OD700 values and the measured OD600–OD700 relationship of the 0% arabinose sample (see A).

D. Effect of FP abundance on OD600 error. The OD600 error was plotted against abundance of each FP in molecules/cell. Grey shading indicates the mean of the samples without arabinose ± 2*standard deviations.