Published in final edited form as:

Methods. 2014 March 15; 66(2): 188–199. doi:10.1016/j.ymeth.2013.07.040.

Maximizing the quantitative accuracy and reproducibility of Förster resonance energy transfer measurement for screening by high throughput widefield microscopy

Fred Schaufele

Center for Reproductive Sciences HSE-1622, 513 Parnassus, University of California San Francisco, San Francisco CA, 94143-0556

Abstract

Förster resonance energy transfer (FRET) between fluorescent proteins (FPs) provides insights into the proximities and orientations of FPs as surrogates of the biochemical interactions and structures of the factors to which the FPs are genetically fused. As powerful as FRET methods are, technical issues have impeded their broad adoption in the biologic sciences. One hurdle to accurate and reproducible FRET microscopy measurement stems from variable fluorescence backgrounds both within a field and between different fields. Those variations introduce errors into the precise quantification of fluorescence levels on which the quantitative accuracy of FRET measurement is highly dependent. This measurement error is particularly problematic for screening campaigns since minimal well-to-well variation is necessary to faithfully identify wells with altered values. High content screening depends also upon maximizing the numbers of cells imaged, which is best achieved by low magnification high throughput microscopy. But, low magnification introduces flat-field correction issues that degrade the accuracy of background correction to cause poor reproducibility in FRET measurement. For live cell imaging, fluorescence of cell culture media in the fluorescence collection channels for the FPs commonly used for FRET analysis is a high source of background error. These signal-to-noise problems are compounded by the desire to express proteins at biologically meaningful levels that may only be marginally above the strong fluorescence background. Here, techniques are presented that correct for background fluctuations. Accurate calculation of FRET is realized even from images in which a non-flat background is 10-fold higher than the signal.

Keywords

Förster resonance energy transfer; fluorescence; androgen receptor; high throughput microscopy; high content analysis

1 Introduction

Förster resonance energy transfer (FRET) occurs when energy excited within a high energy 'donor' fluorophore is non-radiatively transferred to an adjacent 'acceptor' fluorophore [1–

© 2013 Elsevier Inc. All rights reserved.

Address all correspondence to Fred Schaufele, Box 0540, University of California San Francisco, San Francisco, CA, 94143-0556; Tel: (415) 476-7086; fred@diabetes.ucsf.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

4]. Upon FRET, the intensity, lifetime and anisotropy of donor fluorescence change as the acceptor captures some of the donor energy that otherwise would have been emitted as donor fluorescence [5–12]. Anisotropy measurements of FRET are most often conducted using a single fluorophore since the donor and acceptor need not be spectrally different. By contrast, lifetime and intensity-based methods use spectrally distinct fluorophores since the properties of the donor and acceptor fluorophores must be independently measured. Most lifetime-based methods measure the decrease in donor lifetime as energy is transferred to the distinct acceptor fluorophore whereas intensity-based FRET measurements quantify the diminished donor fluorescence intensity, most often relative to the elevated intensity from the distinct acceptor.

The sensitivity of FRET to Angstrom-level differences in the proximities and orientations of fluorophores has made energy transfer between fluorophores an excellent surrogate measurement of the interactions and structures of fluorophore-tagged factors [5, 13–14]. Unlike classic *in vitro* analyses of biochemistry and structure, FRET microscopy of fluorophore-tagged factors and complexes in cells enables determination of the impact of intracellular location, cell physiology or cell type on protein biochemistry and structure. Thus, cellular FRET analysis uniquely may provide highly significant information about biologic function within the context of the complex cell environment.

Despite the advantages of cellular FRET analysis, it has not yet become routine in the biological sciences. Even when FRET measurements are conducted appropriately, a considerable degree of variation in the data collected often limits investigator confidence in the output. Some of the variation may be due to genuine, cell-to-cell variations, which is part of the reason for conducting FRET analysis in cells. Much of the biologic variation also originates from biochemical considerations that affect FRET levels in cells that express different levels of the FP-tagged interacting factors [15–17]. By contrast, the most common non-biologic source of FRET variation originates with measurement errors inherent when quantifying a weak fluorescence signal above a high and variable background. FRET measurement is particularly sensitive to this signal-to-noise issue since the changes in fluorescence lifetime, anisotropy or intensity that accompany FRET typically are small and therefore difficult to accurately quantify.

The requirement for a strong signal above background conflicts with the desire to express in the cell a fluorophore-tagged protein at low ‘physiologic’ levels. Overexpression of the fluorophore-tagged proteins saturates its interactions with the limiting components of the cellular pathway being investigated. Unfortunately, for biologically meaningful FRET studies in living cells of protein structure and biochemistry, in which the target proteins are fused with derivatives of the cyan (donor) and yellow (acceptor) fluorescent proteins (CFP, YFP), a very high level of fluorescence originating from the cell culture media in the relevant imaging channels creates a high noise above which the weak FP signals must be accurately quantified. The quantification of, and accurate correction for, this high background is further impeded by uneven imaging across the field. Those background correction issues are particularly problematic for widefield collections using low magnification objectives with lower numerical apertures (NAs), which are typically used to rapidly collect the large numbers of cellular data points necessary for high content analysis and screening.

Here, we summarize the background correction problems (section 3) and describe (section 4) then validate (section 5) background correction protocols that improve the accuracy of FRET quantification under poor signal-to-noise conditions. The origins of some of the background issues are described and some methods are discussed that have been empirically defined in our laboratory to reduce those issues as much as possible prior to image collection

(sections 2, 3 and 6). Quality control methods to define objects in which the background subtraction protocols have failed are indicated. The results show that application of the background subtraction and quality control methods increases the reproducibility of FRET quantification for poor signals against very high noise.

2 Materials and Methods

2.1 Stable cell line expressing the CFP-AR-YFP probe

The '3-6-1' stable HeLa cell line, described previously [17], was maintained in DME-H21 supplemented with 5% calf serum. G418 and blasticidin were added to 700 µg/ml and 25 µg/ml, respectively, final concentrations in the media. Those drugs enable the growth of only those cells that retain the expression vectors conferring G418 and blasticidin resistance, which were co-integrated into the HeLa cell genome with, respectively, the expression cassettes for the CFP-AR-YFP FRET reporter [18–19] and for the mCherry_{NLS}mCherry nuclear marker [20].

The HeLa cell line was previously estimated to express, on average, ~1400 CFP-AR-YFP reporter proteins in each cell [17]. That low level expression makes this an excellent cell line for establishing methods to improve FRET measurement under poor signal-to-noise conditions. As the amounts of energy transferred from CFP to YFP, attached to the Androgen Receptor (AR), differ when different AR ligands are added to the cell culture media [17, 19], the ability to accurately measure those differences under challenging image collection conditions is the object of the current study.

2.2 Plating reporter cells

Most serum products contain sufficient androgen to activate the AR. Two days prior to plating the cells, the cell culture media used to maintain the cells was removed from the plate with four successive washes of phosphate buffered saline. The 'experimental' media added to the cells contained 5% fetal calf serum charcoal-stripped of steroids (HyClone SH30068.03, Thermo Scientific, Logan, UT, USA) in a 50:50 mixture of phenol-red-free, glutamine-free DME-H21/ Ham's F-12 media supplemented with 0.4 mM glutamine. Two days incubation with the hormone-free media is sufficient to return the AR to its unliganded state.

For plating, cells were removed from the tissue culture dish by trypsin digestion and collected into the androgen-free media. Cells were counted and diluted in androgen-free media typically to 20,000 cells/ml. 40 µl of this suspension (800 cells) were plated per well into 384-well tissue-culture plates (Greiner Bio-One 781091, Frickenhausen, Germany) and centrifuged briefly to plate cells and remove air bubbles. One day after plating the cells, various steroids were diluted into androgen-free media at five times their desired final concentrations. 10 µl of drug mixture was added to the 40 µl in each well. For the current study, multiple wells were treated with each drug to establish the reproducibility of the measurements as a test for accurate background subtraction. The drug-treated cells were incubated overnight. Images were collected as described in section 2.4.

Beyond the significant optical contributors to a non-uniform background (corrected by the methods discussed here), air bubbles represent the most common contributor to a non-uniform background. However, unlike the optical contributions which are relatively consistent and accurately eliminated (section 4.1), the air bubbles, or other fluorescence debris within the system, are sporadic. Cells impacted by those sporadic contributions can be eliminated only by the identification of unusual discrepancies in object measurements using quality control measurements [18](section 5.4).

2.3 Reducing high background levels in the CFP and FRET channels

As will be described in section 3.1, fluorescence backgrounds are extremely high in the channels used to collect CFP and FRET. Examination of background fluorescence levels upon exchanging different components of the study showed that the background originated from multiple sources, none of which can be completely removed. The primary sources of background fluorescence include the media itself, the calf serum and commercial glutamine supplements added to the media (Irina Krylova, F.S., not shown). That background can be removed by exchanging out the complete media for PBS but, since that would also wash out treatments unique to each study, those washing steps are impractical for screening studies. For the images collected in this study, glutamine was lowered to 20% of normal to minimize background fluorescence. Otherwise, the very weak signals emitted from CFP-AR-YFP become even more lost within the background. This signal-to-noise issue will confound any FRET measurement, including those based on fluorescence lifetime, anisotropy or intensity, unless there are ways to accurately parse out the weak signal from the overwhelming background fluorescence. A method will be described and validated that accurately subtracts out background intensities originating from non-cellular sources and thereby improves the reliability of FRET measurement.

The signal-to-noise also can be improved by selecting plate types that provide lower fluorescence in the Donor and FRET channels where high backgrounds plague the FRET analysis of live cells growing in culture media (sections 3.1 to 3.3). Cell culture media was added to six sample plates provided by five manufacturers (Aurora Spectral Technologies #32621, Becton Dickinson Falcon #353362, #354719, Costar Corning #3712, Greiner BioOne #781091, Matrical #MGB101-1-2-LG) and imaged using the collection channels (section 2.4) of the current study (I. Krylova, F.S., unpublished). All six plates showed the very high level of fluorescence in the Donor and FRET channels that originated predominantly with the cell culture media. However, the Aurora, Greiner BioOne and Matrical plates showed noticeably lower fluorescence in those crucial channels. After examining also the plate-specific fluorescence properties in the YFP and mCherry channels, and considering each plate's list price, our preference was for the Greiner BioOne and Matrical plates. The Aurora, Greiner BioOne and Matrical plates also showed superior consistency in the size of the well (at least for the plate lots tested), which ensures that fields are collected from the same position within each well. These tests were conducted only on thin-bottomed 384-well formatted plates. Since the properties tested are likely to vary with other plate formats for each manufacturer, the user is encouraged to conduct similar tests to select a plate type and lot number that provides the lowest noise and the most reproducibility between wells. Overall, the selection criteria for us was biased heavily towards plates on which the desired cell type grew best, followed by the optical considerations and finally by the cost.

2.4 Collection and calculation of FRET

All images were collected on an IXMicro high throughput microscope (Molecular Devices Corp., Sunnyvale, CA, USA) using a 10 $\times$, (0.5 NA), S Fluor objective (Nikon Instruments, Tokyo, Japan). Laser autofocus established the interface of each well bottom with the cell culture media. All fluorescence channels described below were collected at the same offset (4 μ m) above that interface. The offset was selected to place the cell nuclei into optimal focus since that was the subcellular target for fluorescence quantification. The same offset was used for each channel as it is necessary to collect images for FRET analysis from the same focal plane. The rapid laser autofocus protocol also is consistent with the high throughput requirements for screening applications.

The mCherry_{NLS}mCherry nuclear marker was specifically excited with 560–590 nm light and the emissions captured in a 635–675 nm window. YFP and CFP fluorescence amounts were collected from the same field in Acceptor (excite with 498–516 nm/collect with 528–556 nm) and Donor (422–432 nm/457–487 nm) channels. FRET depends on a considerable overlap of the Donor emission spectrum with the excitation spectrum for the Acceptor [5]. This requirement also typically results in a considerable amount of fluorescence, in the FRET channel, from both the independently fluorescing CFP and YFP. The FRET channel (422–432 nm/528–556 nm) therefore contains fluorescence contributions from three sources (YFP excited by 422–432 nm; the ‘yellow’ fluorescence directly emitted from CFP in the 528–556 nm window; and the comparatively small amount of excited CFP energy that is transferred to YFP and emitted in the 528–556 nm window).

The challenge of intensity-based FRET measurement is to accurately measure how much CFP and YFP is present and then to compute and remove the independent contributions of those fluorophores to the FRET channel [5–8]. The methods used to parse out the contributions of independently emitting CFP and YFP to the FRET channel, and to determine what percentage of CFP energy was transferred to YFP and emitted as fluorescence in the FRET channel, were described previously [18, 21]. This intensity-based method of FRET determination, referred to as sensitized emission FRET, therefore relies on the precise quantification of fluorescence in all three Acceptor, Donor and FRET channels. If quantification is poor in even one channel, the quality of the FRET calculation will be severely compromised. Sensitized emission FRET analysis thus is an excellent, highly stringent test for evaluating background subtraction procedures intended to improve measurement accuracy and reproducibility.

Prior to the FRET calculation, background subtraction protocols (sections 4.1, 4.2) removed the fluorescence in Acceptor, Donor and FRET fluorescence that did not originate from CFP and YFP. Background-subtracted fluorescence levels of CFP-AR-YFP intensity and energy transfer were measured within the nuclei of cells (section 5). The nuclei were automatically segmented as areas of contiguous mCherry_{NLS}mCherry fluorescence using the ‘count nuclei’ function within the MetaXpress4 image analysis program (Molecular Devices Corporation). Similar segmentation programs are available from a variety of commercial sources and as ImageJ plug-ins.

The ‘mCherry_{NLS}mCherry’ channel was collected for 20 ms, two ‘Acceptor’ (YFP) channels were collected for 150 ms and 75 ms, two ‘Donor’ channels were collected for 500 ms and 250 ms and two ‘FRET’ channels were collected for 500 ms and 250 ms. Two different integration times were used for the those three channels to 1) determine signal noise levels critical to proper background subtraction (section 4.2) and 2) establish quality controls in which unusual objects, or objects of insufficient intensity to be accurately measured, were eliminated if the background-subtracted fluorescence in the 2× exposure window was not between 1.90 and 2.10 times that of the background-subtracted fluorescence in the 1× exposure window (section 5.4).

3 Theory

3.1 Poor signal-to-noise in CFP and FRET channels

Our laboratory’s studies are oriented towards the high content analysis of interactions in cells grown in multi-well dishes in which each well is treated with a different drug. That goal requires the collection and analyses of millions of cells in thousands of wells [22–26]. The measurements from each well must be highly reproducible. Otherwise, well-to-well variations in FRET levels that originate solely from measurement error will degrade the ability to establish, with confidence, if a drug treatment in a specific well actually changed

FRET [27]. The issues discussed below are considered with this high volume data collection in mind, but also are pertinent to the accurate collection of FRET data under any experimental paradigm.

In our experience, photobleaching is a significant impediment to accurate and reproducible FRET collection that occurs as a manual microscope operator dwells on cells during visual surveillance. In our high throughput studies, cells are not exposed to light until the image is automatically collected, which minimizes the photobleaching issue.

The major hurdle to accurate, FRET measurement occurs with errors in correctly subtracting out the background fluorescence in an image. The background issues are exemplified in Figure 1. Images were collected from a HeLa cell line stably expressing an androgen receptor (AR) tagged at its amino terminus with CFP and at its carboxy terminus with YFP ('CFP-AR-YFP). For cells treated with 10^{-8} M testosterone (the natural AR activating hormone), YFP fluorescence from the AR was localized predominantly to cell nuclei marked with an mCherry-labeled nuclear marker (Fig. 1A, see inset images). In the absence of hormone, the AR was predominantly, but not exclusively, cytoplasmic. This hormone dependence of AR nuclear distribution has been well described previously [19, 28–29].

The cells express under 2,000 CFP-AR-YFP molecules per cell [17]. YFP fluorescence was detected because the fluorescence background from non-cellular sources (Fig. 1A, Media Only) was low in relationship to the signal. Because both CFP and YFP were attached to the same AR, each cell also expressed the same number of CFP molecules as YFP molecules. However, CFP detection was poor (Fig. 1B) compared to YFP. Although CFP also is less bright than YFP [30], the major impediment for imaging CFP was a large amount of fluorescence in the CFP channel (see 2.4 for channel specifications) originating from non-cellular sources (Fig. 1B, Media Only; all CFP, FRET and YFP channel images were scaled identically for display). This high amount of background fluorescence also was present in the FRET channel (Fig. 1C). The ability to obtain accurate and reproducible FRET measurement therefore was challenged by the need to precisely quantify weak CFP and FRET channel fluorescence from the CFP-AR-YFP probe against the strong fluorescence from other sources.

3.2 Improving signal-to-noise

It is recommended to improve signal-to-noise by using newer derivatives or alternatives of CFP, such as Cerulean3, that are up to 2.7 times brighter than the original 'ECFP' derivative [5, 31]. However, even with that improvement, the levels of background fluorescence in the Donor channel would remain well above the Cerulean3 signal and would need to be properly background-corrected if accurate, reproducible FRET measurement were to be realized. Newer, brighter YFP-based Acceptors similarly can improve the signal in the FRET channel against the very high fluorescence background. Ultimately, the problem remains: a very high level of media fluorescence at wavelengths required for the excitation of CFP and its derivatives impede fluorescence quantification of those FPs.

FP fluorescence quantification also can be improved by using higher NA objectives that collect less out-of-focus light (and therefore less media fluorescence) in the optical plane of the cells expressing the FP-tagged factors. Higher NA objectives also improve the uniformity of collection across a field, which represents another background problem that must be overcome for accurate and reproducible FRET measurement (section 3.3). However, the highest NA objectives are those with the highest magnifications, which conflicts with the desire, in screening applications, to collect as many cells as possible in a field. Perhaps the best long-term solution to overcoming the severe background intensities that plague live cell FRET imaging using CFP and YFP derivatives is to use more red-

shifted FPs detected by fluorescent channels that are not as severely contaminated by fluorescence from the media [32]. Regardless, evidence will be provided for a background correction method that is effective even under very challenging quantification conditions.

3.3 Non-uniformity in background across a field

The 'Media Only' images of figure 1 showed also that the center of the field is brighter than those around the margins. The extent of that non-uniformity is best exemplified visually by scaling a 'Media Only' image such that the lowest intensity pixels are displayed as black (Fig. 2A, CFP channel shown). The lack of a uniform background presents another challenge for the accurate quantification of CFP-AR-YFP fluorescence levels necessary for reproducible intensity measurement and FRET calculation.

The average fluorescence intensities at each pixel in a diagonal trace (Fig. 2A, pink line) across a field are shown for the Acceptor, Donor and FRET channels (Fig. 2B). The traces across fields from wells in which media was added without cells ('media & plate fluorescence + noise') was compared to traces from images collected when the objective was put in the exact same position but without any plate or media. The latter image collects camera noise and ambient light in the system, which is collectively referred to as 'noise' in the next sections. Noise also may be affected by the plate or other additional experimental elements present at image collection. Methods to specifically determine the noise (section 4.2) under the actual collection conditions are a key part of the background subtraction method.

Collection conditions to reduce the non-uniformity are described in sections 6.1 and 3.2, but cannot be eliminated in most widefield images. As a result, images from cells located in the margins of a field will be subject to different local backgrounds than those in the center of the field. The challenge for any precise fluorescence quantification is to eliminate that non-uniform background to obtain accurate fluorescence quantification. The FRET measurement methods described here appropriately account for and eliminate that non-uniform background.

3.4 Simplistic background subtraction

The Media Only images of figures 1 and 2 represent the amounts of fluorescence when no cells are plated. The Media Only images were collected in wells parallel to those in which cells were plated in the same media and grown under the same conditions. In theory, those Media Only images should represent the background that partially obscures the fluorescence from the FP.

Most cells possess intrinsic fluorophores that autofluoresce in the Donor and FRET channels used for measuring FRET between CFP and YFP. The nucleus, in which all quantification was collected for the current study, has no apparent cellular autofluorescence in those channels. Thus, for the current study, the Media Only images represent the proper backgrounds against which to quantify fluorescence. By contrast, autofluorescence surrounding the nucleus is observed in many cell lines and offers other challenges for removal (see section 6.1 in the Discussion).

A FRET channel image for a cell-containing field (Fig. 3A) is shown together with the pixel intensities along a diagonal line across that image (Fig. 3B, dark gray). A similar diagonal trace of pixel intensities across a background image is shown (light gray); the background image was averaged from multiple 'media only' images in different wells (section 5.1). The intensities along the diagonal trace of the background image parallel the intensities of the cellular image in areas where no cells are present. Thus, subtracting the intensity at each pixel within the background image from the matching pixels within cellular image should

provide background-subtracted fluorescence intensities (Fig. 3B, black) that represent the actual amounts of fluorescence originating from the CFP-AR-YFP reporter.

The simplistic background subtraction approach is valid only if the background traces are identical in each and every well. In practice, the backgrounds vary slightly in intensity from well to well, which prevents the simplistic background correction from being successfully applied. Thus, three different background images of slightly different intensities will cause different degrees of under-subtraction or over-subtraction of background (Fig. 3C). Those well-to-well differences in background level therefore must be accounted for when subtracting backgrounds. Measurements of the extent to which non-cellular areas of an experimental image have different intensities than the same regions present in the background image are described in section 3.5. Those discrepancies, once measured, are used to adjust the background image to a level equivalent to that present in the experimental image (sections 3.6, 4.1, 4.2), which then can be used to eliminate the background from the experimental image.

3.5 Uniform adjustment to simplistic background subtraction

To improve upon the simplistic background correction, it is possible to measure the discrepancy in background between the cellular and background image then adjust the final fluorescence by that difference. The simplest method of accomplishing that adjustment is outlined in figure 4. A background image (Fig. 4A) and an identically collected image of a well plated with CFP-AR-YFP-expressing cells (Fig. 4B) are displayed in a pseudo-colored format to more readily show the image areas with little to no cellular fluorescence.

A ‘mask’ of the areas within the cellular image where no cells are present is created to first identify the locations of cells. Procedures that define those ‘background’ areas where no cells or other objects are present must take into account the different background intensity levels in different areas of the field. Therefore, objects brighter than the immediately adjacent pixels first are defined with automated image segmentation software (Fig. 4C). For this step, the ‘local threshold’ intensity is set very low (typically 5 above the local background on a 12-bit scale) since the objective is to identify even weak debris as an object. Those segmented objects then are expanded (typically by five pixels, Fig. 4D) to ensure that all objects defined are well within the margins of the segmentation map. Once the locations of the expanded objects are defined, a mask is created of the ‘background’ areas where no objects are present (white area in Fig. 4D).

The average intensity of fluorescence under the background mask is determined in both the cellular image and in the background image. That intensity difference describes the extent to which the average background pixel intensity in the background image is above or below that in the cellular image. That intensity correction then is added back to, or subtracted from, each pixel across the entire cellular measurements to arrive at background-subtracted fluorescence intensities that better approximate the actual intensities within the cell.

3.6 Deficiencies in uniform background adjustment

The simplistic background subtraction adjustment (section 3.5) assumes that the fluorescence differences in the background areas of the cellular and background images are of uniform intensity across the field. However, if comparing two background images from adjacent wells that have slightly different intensities, the absolute intensity discrepancy between the two curves is more pronounced in the center of the images (Fig. 5A). Indeed, the difference between those images is higher in the center of the image than at the margins (Fig. 5B).

The simplistic background adjustment protocol (section 3.5) therefore operates under the false assumption that intensity differences in the cellular and background images are uniform across the field. The quantification of fluorescence following a uniform background correction thus introduces measurement errors that vary with field position. Whether one needs a more advanced protocol that more precisely corrects for that position error depends upon how strong the signal is relative to the error. If, for example, the signal intensity is 1000 units above background when the background varies only 10 units across the field, the error is negligible. In that example, the uniform background subtraction adjustment protocol would introduce, at most, a 1% error which is acceptable for most applications.

The goal here was to define background subtraction methods that permitted the proper subtraction for very challenging images in which the background intensity was higher than the signal intensity. Protocols were developed that can properly correct for the discrepancies in background differences across the field. That adjustment is based upon the observation that the fluorescence differences between the experimental and background images are proportional to background pixel intensities throughout the field (Fig. 5C). The proportional corrections described in section 4 are valid (section 5) when applied specifically to the non-uniform components of the intensity signal.

4 Calculations

4.1 Proportional background adjustment

As with the uniform adjustment procedure (section 3.5), the starting point for conducting a proportional background adjustment is to measure the average fluorescence intensities under the background mask (I_{mask}) (Fig. 4D) in both the background image (BKGD; i.e. Media Only average image) and in the experimental image (EXP; i.e. images with cells). However, the difference in background measurement between the background and experimental images is considered proportionally to the uneven background fluorescence. That proportional difference is used to adjust the background image to the intensity of the background present in the experimental image such that the background can be subtracted. Some caveats of this procedure that make it particularly apt for FRET calculation, but that remain limiting for some other fluorescence quantification applications, are discussed in section 6.2.

When conducting the proportional background adjustment, one has to consider that the intensities under the background mask consist of two primary components:

I_n : noise, mostly from the camera and stray light, that is relatively uniform across the field (Fig. 2B) and therefore not subject to proportional adjustment, and

I_f : intensities from fluorescent sources that are non-uniform across the field and result from the uneven illumination more pronounced with low NA objectives and large-chip cameras.

The step-wise procedure for defining, and subtracting, the proportional non-uniform background levels from an experimental image are defined below. The experimental validation of this procedure is presented in section 5.

1. The average intensities under the mask for both the background and experimental images are determined. The noise intensity (determined as described in section 4.2) is subtracted from the average intensities to obtain the fluorescence intensities of the non-uniform components of the background (Eq. 1a) and experimental (Eq. 1b) images.

$$I_{f(BKGD)} = I_{mask(BKGD)} - I_{n(BKGD)} \quad \text{Eq. 1a}$$

$$I_{f(EXP)} = I_{mask(EXP)} - I_{n(EXP)} \quad \text{Eq. 1b}$$

2. A noise-corrected background image (BKGD_{-n}) is created by subtracting the noise intensity level from every pixel across the background image. The resulting image represents the non-uniform component of the background (Eq. 2) that, because of slight well-to-well variations, has a different intensity (Eq. 1a) than that present in the experimental image (Eq. 1b).

$$BKGD_{-n} = BKGD - I_{n(BKGD)} \quad \text{Eq. 2}$$

3. The proportional discrepancy in the intensities of the non-uniform backgrounds in the noise-corrected background and experimental images must be accounted for (Fig. 5). The relative proportion of noise-subtracted fluorescence under the mask in the background and experimental images is expressed as a correction factor (k):

$$k = I_{f-EXP} / I_{f-BKGD} \quad \text{Eq. 3}$$

4. Multiplying the correction factor (Eq. 3) across the entire noise-subtracted background image (Eq. 2) creates a correction image (CORR) that is proportionally adjusted to background level present in the experimental image. The adjusted background image is to be used for background correction of the experimental image.

$$CORR = k \times BKGD_{-n} \quad \text{Eq. 4}$$

5. Since the corrections are based upon noise-corrected images, a noise-corrected experimental image (EXP_{-n}) must first be created by subtracting the noise intensity level from every pixel across the experimental image

$$EXP_{-n} = EXP - I_{n(EXP)} \quad \text{Eq. 5}$$

6. The noise-corrected background image (Eq. 4) then is subtracted from the noise-subtracted experimental image (Eq. 5) to create the background-subtracted experimental image, X, that is flat-field corrected across the entire image, with the background reduced to zero.

$$X = EXP_{-n} - CORR \quad \text{Eq. 6}$$

This proportional background adjustment procedure is conducted for each of the Acceptor, Donor and FRET channels required to calculate the FRET level. Background (Media Only) images collected for each channel are used to conduct the proportional adjustments since the uneven background is shaped differently for each channel (Fig 2B). An example of the success of that procedure is provided in figure 6 in which the non-uniform background fluorescence along the diagonal across all three channels (Acceptor, Donor, FRET) is reduced by the proportional background subtraction process (black line) to zero except where cells are present. Further validation of the background-subtraction process will be

presented in section 5 by showing the exquisite reproducibility of FRET measurements under these very challenging signal-to-noise conditions.

4.2 Determination of noise

Not discussed in section 4.1 is the measurement of noise. In two images of the same field, collected at 1× and 2× exposure times, the fluorescence intensity captured in the 2× exposure time should be twice that of the 1× exposure (provided that the camera is quantitatively linear) [18]. The presence of any underlying noise would offset the intensities captured in both exposure times and cause the total signal to deviate from the expected 2× fluorescence. The amount of that noise is easily approximated by plotting the intensity of fluorescence under the background mask against the exposure times for the two images (Fig. 7). The y-intercept represents the baseline intensity not affected by the exposure time. That y-intercept was used as the noise variable in all of the background-correction calculations shown in figures 5C, 6 and 8.

The noise obtained from the y-intercept in multiple studies of cell-containing images was compared against the background intensities obtained when just exposing the microscope chamber (with no cells or media) for the same integration times (Table 1). The calculated noise approximates reasonably well that obtained by simple exposure of air, particularly in the CFP and FRET channels. The calculated noise in the YFP channel was consistently higher in the experimental images than the ‘in air’ images. It is likely that noise originating from stray light is altered by the presence of the plate and/or the media within the wells. Note also that, within an experiment, the noise determinations are extremely consistent for each channel (typically $\pm <0.5$ units) and that the variations reported in Table 1 reflect different noise levels in different experiments. Because of that consistency, it is possible to establish the noise for each channel from a limited number of control wells in an experiment and then avoid the time-consuming collection of two different exposures for the remaining wells. However, in the current studies, we still collected the different exposure times for each channel since that information was used for our quality control determinations (section 5.4). The empiric success in using this noise detection method, particularly for obtaining highly reproducible FRET measurements under difficult imaging conditions (section 5), provides a strong indication that the methods are valid.

5 Results

5.1 Experimental design

The proportional background adjustment method was tested by examining the reproducibility in fluorescence measurement of CFP-AR-YFP response to different ligands (Fig. 8). The CFP-AR-YFP reporter cell line was seeded into a row of 24 wells within a 384-well plate. Triplicate wells were treated with one of seven ligands (see Fig. 8 legend), including six steroids within the androgen family and one steroid (aldosterone) that is not a ligand for the AR. Aldosterone treatment acts as the negative control. Testosterone treatment was done in duplicate (six wells total). The effect of each ligand on the amounts (YFP fluorescent intensities) of AR in cell nuclei (section 5.2) and on AR structure (FRET from CFP to YFP, section 5.3) was established following background subtraction (section 4) and quality control surveillance (section 5.4).

One day following treatment, two fields were captured in each well. Each field was collected in seven channels (section 2.4): one channel detected the mCherry_{NLS}mCherry nuclear marker and two channels each (at 1× and 2× exposure times) detected the Acceptor (YFP), Donor (CFP) and FRET emissions from the CFP-AR-YFP reporter. For each field, each of the seven channels was segmented to create background masks within the field

(section 3.5). A single background mask was created that contained the background regions common to all channels. Since all seven channels were measuring essentially the same cells, the background locations for each channel overlapped considerably and differed only when some fluorescent debris was present in one channel but not another.

The intensities under the background mask created for each field were determined in the seven channels. That field-specific mask also was applied to the seven background images created for comparison to all wells within the study. Those background images were collected from another row of wells that had been plated with media only (no cells). Fields of Media Only images with any debris in any fluorescent channel were identified by visual inspection and discarded. The intensities at every pixel in Media Only images were averaged to create the mean background images for each of the seven channels (see Fig. 1 for some examples).

The intensities measured under the background mask in the experimental and background control images were used, as described in sections 4.1 and 4.2, to remove the fluorescent background in the Acceptor, Donor and FRET channels of the cellular images. Background was subtracted from the mCherry_{NLS}mCherry channel using the uniform background adjustment (section 3.5), which was appropriate for this red-shifted channel because the background was flat across the field (not shown).

5.2 Ligand-regulated AR translocation into cell nuclei

Within each field, the mCherry_{NLS}mCherry nuclear marker was used to identify cell nuclei. The background-subtracted intensities in each nucleus in each field were recorded for each of the seven channels. The amounts of AR within each nucleus were averaged from all cells in each field as the mean background-subtracted intensities in the Acceptor (YFP) channel. Since the FRET values vary substantially with ligand (section 5.3), Donor (CFP) channel intensities that vary with different levels of FRET are not appropriate for comparing the effects of ligands on AR levels in cell nuclei.

Fig. 8A shows the YFP fluorescence intensities averaged ($\pm$ sd) from all fields in response to each ligand. When no AR ligand was present (Aldo), there was a baseline level of AR within the cell nucleus. As has been well-described previously [19, 28–29, 33], the amounts of AR in the cell nucleus increased strongly following incubation with the strongly androgenic hormones testosterone (T) and dihydrotestosterone (DHT). Also examined here were four other steroids that are immediate precursors to T and DHT along the natural steroid synthesis pathway. Those androgen intermediates (A-diol, DHEA, A-dione, Androst; see Fig. 8 legend for full names) showed varying degrees of ability to elevate the amounts of AR in cell nuclei.

5.3 Ligand-regulated folding of AR in cell nuclei

As shown previously using the CFP-AR-YFP reporter transiently expressed at higher levels in cells, the addition of testosterone changes also the amount of energy transfer [19] (Fig. 8B). Prior to androgen binding, no energy transfer is detected. Thus, even though attached to the same molecule in the CFP-AR-YFP reporter, the FPs are insufficiently close to enable energy transfer.

One day after androgen addition, energy transfer is readily measured and arises predominantly from a well-characterized interaction of the amino terminus of the AR with a surface that forms on the androgen-bound carboxy terminal domain of the AR. That ‘folding’ occurs throughout the cell and brings the two FPs into close proximity to permit energy transfer [19]. Dimerization of the AR, measured either by CFP-AR interaction with AR-YFP or by CFP-AR-CFP interaction with YFP-AR-YFP [19], also occurs,

predominantly in the cell nucleus. However, the energy transfer levels measured seem to be governed almost exclusively by the folding of the AR as 1) shortly after androgen addition, the energy transfer efficiency is similar for the monomeric CFP-AR-YFP reporter in the cell cytoplasm and for the dimeric CFP-AR-YFP reporter in the cell nucleus and 2) mutants that disrupt the AR folding still dimerize but with energy transfer efficiencies similar to those achieved by the rapid folding [19].

Most significantly, figure 8B shows that the FRET measurements obtained under these very challenging signal-to-noise conditions were highly reproducible across all fields treated with a given hormone. For high throughput studies in which thousands of wells are treated each with a unique compound or siRNA, there is little room for duplicating the time-consuming and costly screens. Therefore, the emphasis is on the development of assays that are sufficiently reproducible from well-to-well such that, when a well measurement deviates from the average, there is a reasonable expectation that the deviation will not arise by chance measurement error ('false positive'). Conversely, well-to-well reproducibility is desired to minimize measurement errors in which a well containing a drug that alters FRET is not identified because of measurement errors ('false negative').

The industry standard by which an assay generally is judged as sufficiently reproducible for use in a screen is described by the Z'-factor score [27]. Essentially, if the difference between the mean measurement of treated and sham-treated wells is equal to or greater than the sum of three times the standard deviations for each of the 'no treatment' and 'treatment' wells, the Z'-factor score is an acceptable 0.5 or greater. Assays that meet that criterion are considered worthy of adoption for screening. The Z'-factor score for the FRET measurement between aldosterone and testosterone-treated wells was an exceptional 0.75. As summarized in Fig. 8C, the responses of many AR ligands studied would have met the 0.5 criterion, even with the limited number of wells examined in the current pilot study. The proportional background adjustment methods implemented therefore provided an extremely high level of FRET measurement accuracy even though the signal in the CFP and FRET fields was mostly buried in a non-uniform background.

5.4 Measurements at the margin of detection: quality control measurements

The measurements were highly reproducible (Fig. 8), in part, also because of the implementation of quality controls to screen out objects in which the background-subtracted fluorescence levels were deemed inaccurate. For the Acceptor, Donor and FRET channels, two different exposure times (1× and 2×) were collected to determine the noise (section 4.2) necessary for proportional background adjustment (section 4.1). In addition, duplicate channel collections were used to determine if any given background-adjusted intensity measurement deviated substantially from the 2:1 intensity ratios expected for any of the Acceptor, Donor or FRET channels.

Figure 8D shows the mean percentage of objects classified as sufficiently 'accurate' for inclusion in the FRET analysis (having 2× exposure/1× exposure ratios between 1.9 and 2.1 in all three of the Acceptor, Donor and FRET channels). When the amounts of probe in the cell nucleus were strong (Fig. 8A, T, DHT, A-diol), most cells met that rigorous inclusion criteria. However, with steroids that provided progressively less CFP-AR-YFP in the cell nucleus, the percentage of cells in which the measured intensities were deemed to be sufficiently accurate for FRET analysis decreased in parallel. Thus, the proportional background adjustment provided an excellent correction for background and, when faced with increasingly difficult measurements, the quality control corrections enabled by the protocol can limit measurements only to those objects that are accurately measured.

6 Discussion

6.1 Improving quantitative accuracy through background adjustment

The purpose of most high throughput imaging is to maximize the number of wells collected while obtaining sufficient objects to reliably quantify drug- or siRNA-induced alterations in specific wells. Lower magnification objectives therefore are most commonly used for high throughput campaigns. However, those objectives are more prone than their higher magnification cousins to the vignetting around the edges introduced predominantly by uneven illumination (Fig. 2A). Recent movements to systems that provide an even larger field of view will be even more susceptible to the vignetting problems that must be adequately corrected to obtain sufficient quantitative accuracy.

One can try to overcome that problem through image collection procedures that minimize the non-uniformity. For example, the red-shifted channel used to collect the mCherry-based nuclear marker has a completely flat background, mostly because of the lack of strong, non-uniform media fluorescence above the uniform noise. Based upon that, we previously developed ‘bar-coding’ procedures that enable different red-shifted fluorophores to be distinguished and quantified in red-shifted channels [20] using the uniform background adjustment described in section 3.5. Alternatively, higher quality objectives can help to reduce the non-uniform background. Most of those tend to be higher magnification objectives which are at odds with the desire in high content studies to maximize cell number in a field.

Laser scanning confocal imaging, in which adjacent points are imaged and stitched together, will provide a uniform background across the field. Confocal imaging also restricts image collection to an optical plane that, for cells completely within that plane, will come primarily from the interior of the cell where only the fluorescent probe (and endogenous fluorescent molecules) will be captured without contaminating media fluorescence. Thus, confocal imaging systems may reduce the background collection problem. However, it has been our experience that FRET measurements are far more consistent on camera-based widefield systems than on PMT-based confocal systems, possibly because of quantitative differences in the detectors combined with the comparatively lower photobleaching of fluorophores collected in the widefield system.

The background adjustment procedures described in this report were particularly effective (Fig. 8) for measuring poor signals above a high, but predictable background. However, the procedures may be ineffective in some instances. For instance, the procedures rely on determining the difference in intensities within background areas of the ‘experimental’ image with the same area within the reference ‘background’ image. If the cells are completely confluent throughout the field, which may be dictated by the biology of some studies, this background correction method will not be effective.

Background subtraction issues also arise when background contributions vary unpredictably within a cell or from cell to cell. Cytoplasmic autofluorescence represents such a source of unpredictable background that can be difficult to compensate for, particularly when the FP fluorescence is marginally above a strong autofluorescence signal. Under those challenging conditions, spectral unmixing algorithms are recommended to separate the composite emission spectrum of all fluorophores into its component parts [34–35]. It remains to be established whether the background subtraction methods described here can improve the spectral unmixing process and/or the quantification of the unmixed components. But, for high throughput studies, it is preferable to avoid the increased time required for spectral imaging. The background correction methods presented here thus were validated for high throughput imaging in cell nuclei where cellular autofluorescence is minimal, but

background correction challenges under other conditions remain that can degrade fluorescence quantification and FRET calculation.

6.2 Flatfield correction

The exceptional consistency in FRET measurement achieved using the proportional background adjustment procedures demonstrated the ability to correct accurately for a high level background non-evenly distributed across the field. The two key elements of the procedure included a) the method to separate the uneven component of the signal from the uniform noise and b) the calculation of the proportional adjustment constant only from areas within the field where cells or other objects were not present in the experimental image. The proportional difference between the reference 'background' image and the experimental image thus determined was used to adjust the background image to the background intensities present in the experimental image.

Once adjusted to the intensities in the experimental image, the uneven background is readily subtracted. However, the correctly background-subtracted intensities within each cell nucleus will not have been corrected for the uneven illumination that causes the non-uniform fluorescence. Those measurements thus still will vary predictably (as with the background adjustment procedure) for each location within the field. We did not perform further adjustments for the FRET measurement because FRET is calculated from the relative fluorescence intensities, not the absolute intensities, in the three collection channels at each location within the field. Similarly, our bar-coding live processes, which also are based upon relative, correctly background-subtracted fluorescence intensities in different emission channels [20], can be expanded with the procedures described in section 4 to fluorescence channels, such as the 'green' channels, that have substantial, non-uniform backgrounds arising from the cell culture media. In addition, flat-field determinations of absolute background-subtracted intensity at each location within a field can be achieved by adding steps to correct the background-adjusted cell intensities for their locations within the field. That involves little more than creating a mask over the cell nuclei (or any other object to be measured), then comparing their intensities in the background-adjusted image (section 4-1, step 6) against the noise-subtracted background image in a fashion similar to that described in steps 2–6.

Other processes may be used to adjust the intensities within objects for their local background that vary throughout a field. One crude method is to measure the intensities of an area in a 'ring' around the object. However, if the ring area captures 'background' areas containing fluorescence above the real background, the background will be over-subtracted and errors will be introduced. Many high throughput instruments come with pre-packaged commercial background flattening applications, for which the underlying process is often not well-described. The high throughput instrument used for the current studies comes with a flatfield correction application that, when applied to the difficult images shown here, did not completely adjust the background to a uniform intensity across the field. By contrast, the detailed proportional adjustment methods described here, provided both excellent flatfield correction and background subtraction. Ideally, if flatfield correction procedures are available that accurately create a uniform background in which the fluorescence values within cells located in different areas of the field are equivalently changed, then one can apply the uniform background adjustment (section 3.5) to the flatfield-corrected experimental and background images to obtain quality fluorescent intensity measurements.

6.3 Steroid intermediates in AR action

Prior studies of intramolecular AR folding, collected on other microscopes, also indicated that the amount of energy transfer in the absence of hormone was near zero and increased to

40% upon testosterone binding. Therefore, the energy transfer values obtained with the proportional background adjustment procedure not only were highly reproducible from field to field, but they also agreed with the prior findings. The current studies also examined other steroids that are precursors in the endogenous synthetic pathway leading to testosterone and dihydrotestosterone. Those precursors have only marginal chemical differences from the fully active androgens. In general, the precursors had variable effects that, for each precursor, were similar on two different measures of AR activity: AR nuclear translocation (Fig. 8A) and AR structural folding (Fig. 8B).

Overall, the precursors ranged from being fully capable to completely inactive AR regulators. DHEA, which is heavily marketed as a dietary supplement, was completely inert even at the high doses used here (10^{-6} M). Androstenedione, which had been heavily abused within the athletic community (particularly in American baseball) prior to its listing as an illegal drug, had mild actions at the AR in both assays. Of course, androstenedione and DHEA, as precursors to testosterone, may have greater effects through conversion in the body into biologically active androgens or through actions at other proteins.

Because of such physiologic activities, the AR is a clinically important target. Major drug screening efforts are designed to define novel androgen analogs that activate only specific AR activities and to identify AR antagonists that shut down AR action [36–39]. Multiple different types of AR read-outs have been used [33, 40–41], including the CFP-AR-YFP folding FRET assay [42–43] used here. In those prior folding screens, the background issues were overcome by fixing the cells and reading the output in PBS. We note that, in our own experience, the intramolecular FRET read-out from fixed cells degrades with time, probably because fixation does not weld a protein into a specific conformation and the subsequent washing away of the hormone leads to a progressively diminished FRET measurement. We anticipate that the capabilities described here, enabled by the development of the accurate background-subtraction procedures, will permit a better quantification of intramolecular FRET in conjunction with multiple other AR activities read in high content screens on FP-tagged ARs.

7 Conclusions

For the challenging conditions of detecting a tracer-level fluorescent probe against a high background, there is a need for precise background correction. A highly accurate background adjustment method was developed and validated in studies of androgen receptor action. The method will improve the quantitative measurement of any fluorescence and will be of particular benefit to studies in which a very weak signal is being investigated against a very high background.

Acknowledgments

The background subtraction procedure was developed for a variety of FRET-based, high throughput studies funded by the US Department of Defense's Prostate Cancer Research Program (PC080258), the Partnership for Clean Competition, and an Early Exploration Award from the Novo Nordisk Diabetes Innovation Award Program. Personal funds (F.S.) also supported this program. Maintenance of microscopy equipment was partially offset with Center grant support from the National Institutes of Health (P30 DK63720).

Abbreviations

FRET	Förster resonance energy transfer
FP	fluorescent protein

AR androgen receptor
NA numerical aperture

Literature Cited

1. Förster T. Zwischenmolekulare energiewanderung und fluoreszenz. *Ann Phys.* 1948; 6:54–75.
2. Förster T. Transfer mechanisms of electronic excitation. *Discuss Faraday Soc.* 1959; 27:1–17.
3. Sun Y, Wallrabe H, Seo SA, Periasamy A. FRET microscopy in 2010: The legacy of theodor förster on the 100th anniversary of his birth. *Chemphyschem.* 2011; 12:462–474. [PubMed: 21344587]
4. Förster T. Energy migration and fluorescence. 1946. *J Biomed Opt.* 2012; 17:011002. [PubMed: 22352636]
5. Day RN, Davidson MW. Fluorescent proteins for FRET microscopy: Monitoring protein interactions in living cells. *Bioessays.* 2012; 34:341–350. [PubMed: 22396229]
6. Elangovan M, Wallrabe H, Chen Y, Day RN, Barroso M, Periasamy A. Characterization of one- and two-photon excitation fluorescence resonance energy transfer microscopy. *Methods.* 2003; 29:58–73. [PubMed: 12543072]
7. Gordon GW, Berry G, Liang XH, Levine B, Herman B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys J.* 1998; 74:2702–2713. [PubMed: 9591694]
8. Koushik SV, Chen H, Thaler C, Puhl HL, Vogel SS. Cerulean, venus, and venusy67c FRET reference standards. *Biophys J.* 2006; 91:L99–L101. [PubMed: 17040988]
9. Piston DW, Rizzo MA. FRET by fluorescence polarization microscopy. *Methods Cell Biol.* 2008; 85:415–430. [PubMed: 18155473]
10. Day R, Schaufele F. Imaging molecular interactions in living cells. *Mol Endocrinol.* 2005; 19:1675–1686. [PubMed: 15761028]
11. Sun Y, Hays NM, Periasamy A, Davidson MW, Day RN. Monitoring protein interactions in living cells with fluorescence lifetime imaging microscopy. *Methods Enzymol.* 2012; 504:371–391. [PubMed: 22264545]
12. Zeug A, Woehler A, Neher E, Ponimaskin EG. Quantitative intensity-based FRET approaches—a comparative snapshot. *Biophys J.* 2012; 103:1821–1827. [PubMed: 23199910]
13. Vogel S, Thaler C, Koushik SV. Fanciful FRET. *Sci STKE.* 2006; 2006(331):re2. [PubMed: 16622184]
14. Day RN, Periasamy A, Schaufele F. Fluorescence resonance energy transfer microscopy of localized protein interactions in the living cell nucleus. *Methods.* 2001; 25:4–18. [PubMed: 11558993]
15. Chen H, Puhl H 3rd, Ikeda S. Estimating protein-protein interaction affinity in living cells using quantitative FRET resonance energy transfer measurements. *J Biomed Opt.* 2007; 12:054011. [PubMed: 17994899]
16. Hoppe A, Christensen K, Swanson JA. Fluorescence resonance energy transfer-based stoichiometry in living cells. *Biophys J.* 2002; 83:3652–3664. [PubMed: 12496132]
17. Kofoed EM, Guerbador M, Schaufele F. Structure, affinity, and availability of estrogen receptor complexes in the cellular environment. *J Biol Chem.* 2010; 285:2428–2437. [PubMed: 19926790]
18. Kofoed E, Guerbador M, Schaufele F. Dimerization between aequorea fluorescent proteins does not affect interaction between tagged estrogen receptors in living cells. *J Biomed Opt.* 2008; 13:031207. [PubMed: 18601531]
19. Schaufele F, Carbonell X, Guerbador M, Borngraeber S, Chapman M, Ma A, Miner J, Diamond M. The structural basis of androgen receptor activation: Intramolecular and intermolecular amino-carboxy interactions. *Proc Natl Acad Sci U S A.* 2005; 102:9802–9807. [PubMed: 15994236]
20. Krylova I, Kumar RR, Kofoed EM, Schaufele F. A versatile, bar-coded nuclear marker/reporter for live cell fluorescent and multiplexed high content imaging. *PLoS One.* 2013; 8:e63286. [PubMed: 23691010]

21. Chen H, Puhl HL, Koushik SV, Vogel SS, Ikeda SR. Measurement of fret efficiency and ratio of donor to acceptor concentration in living cells. *Biophys J.* 2006; 91:L39–L41. [PubMed: 16815904]
22. Carpenter A. Image-based chemical screening. *Nat Chem Biol.* 2007; 3:461–465. [PubMed: 17637778]
23. Carpenter AE. Extracting rich information from images. *Methods Mol Biol.* 2009; 486:193–211. [PubMed: 19347625]
24. Denner P, Schmalowsky J, Pechtl S. High-content analysis in preclinical drug discovery. *Comb Chem High Throughput Screen.* 2008; 11:216–230. [PubMed: 18336214]
25. Korn K, Krausz E. Cell-based high-content screening of small-molecule libraries. *Curr Opin Chem Biol.* 2007; 11:503–510. [PubMed: 17931958]
26. Zock JM. Applications of high content screening in life science research. *Comb Chem High Throughput Screen.* 2009; 12:870–876. [PubMed: 19938341]
27. Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen.* 1999; 4:67–73. [PubMed: 10838414]
28. Georget V, Lobaccaro J, Terouanne B, Mangeat P, Nicolas J, Sultan C. Trafficking of the androgen receptor in living cells with fused green fluorescent protein-androgen receptor. *Mol Cell Endocrinol.* 1997; 129:17–26. [PubMed: 9175625]
29. Tyagi RK, Lavrovsky Y, Ahn SC, Song CS, Chatterjee B, Roy AK. Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. *Mol Endocrinol.* 2000; 14:1162–1174. [PubMed: 10935541]
30. Day RN, Davidson MW. The fluorescent protein palette: Tools for cellular imaging. *Chem Soc Rev.* 2009; 38:2887–2921. [PubMed: 19771335]
31. Markwardt ML, Kremers GJ, Kraft CA, Ray K, Cranfill PJ, Wilson KA, Day RN, Wachter RM, Davidson MW, Rizzo MA. An improved cerulean fluorescent protein with enhanced brightness and reduced reversible photoswitching. *PLoS One.* 2011; 6:e17896. [PubMed: 21479270]
32. Lam AJ, St-Pierre F, Gong Y, Marshall JD, Cranfill PJ, Baird MA, McKeown MR, Wiedenmann J, Davidson MW, Schnitzer MJ, Tsien RY, Lin MZ. Improving fret dynamic range with bright green and red fluorescent proteins. *Nat Methods.* 2012; 9:1005–1012. [PubMed: 22961245]
33. Marcelli M, Stenoien D, Szafran A, Simeoni S, AgoulNIK I, Weigel N, Moran T, Mikic I, Price J, Mancini M. Quantifying effects of ligands on androgen receptor nuclear translocation, intranuclear dynamics, and solubility. *J Cell Biochem.* 2006; 98:770–788. [PubMed: 16440331]
34. Thaler C, Koushik SV, Blank PS, Vogel SS. Quantitative multiphoton spectral imaging and its use for measuring resonance energy transfer. *Biophys J.* 2005; 89:2736–2749. [PubMed: 16040744]
35. Ecker RC, de Martin R, Steiner GE, Schmid JA. Application of spectral imaging microscopy in cytomics and fluorescence resonance energy transfer (fret) analysis. *Cytometry A.* 2004; 59:172–181. [PubMed: 15170596]
36. Lamont KR, Tindall DJ. Minireview: Alternative activation pathways for the androgen receptor in prostate cancer. *Mol Endocrinol.* 2011; 25:897–907. [PubMed: 21436259]
37. Taplin M. Drug insight: Role of the androgen receptor in the development and progression of prostate cancer. *Nat Clin Pract Oncol.* 2007; 4:236–244. [PubMed: 17392714]
38. Attard G, Richards J, de Bono JS. New strategies in metastatic prostate cancer: Targeting the androgen receptor signaling pathway. *Clin Cancer Res.* 2011; 17:1649–1657. [PubMed: 21372223]
39. Lonergan PE, Tindall DJ. Androgen receptor signaling in prostate cancer development and progression. *J Carcinog.* 2011; 10:e20.
40. Hartig SM, Newberg JY, Bolt MJ, Szafran AT, Marcelli M, Mancini MA. Automated microscopy and image analysis for androgen receptor function. *Methods Mol Biol.* 2011; 776:313–331. [PubMed: 21796534]
41. Szafran A, Szwarc M, Marcelli M, Mancini M. Androgen receptor functional analyses by high throughput imaging: Determination of ligand, cell cycle, and mutation-specific effects. *PLoS ONE.* 2008; 3:e3605. [PubMed: 18978937]

42. Jones J, An W, Diamond M. Ar inhibitors identified by high-throughput microscopy detection of conformational change and subcellular localization. *ACS Chem Biol.* 2009; 4:199–208. [PubMed: 19236099]
43. Jones J, Diamond M. A cellular conformation-based screen for androgen receptor inhibitors. *ACS Chem Biol.* 2008; 3:412–418. [PubMed: 18582038]

Highlights

- A method is described to accurately subtract background in intensity-based images
- The method is successful under highly challenging measurement conditions
- High throughput FRET determination of androgen receptor structure is enabled
- Different steroids had similar effects on different androgen receptor activities

Fig. 1. Quantification of fluorescence and FRET from trace levels of CFP-based probes is impeded by high backgrounds in Donor and FRET channels

A, An androgen receptor (AR) dual tagged with CFP and YFP is imaged by YFP fluorescence in the 'Acceptor' channel. Incubation with an androgen (testosterone) redistributes the AR from a predominantly cytoplasmic to a predominantly nuclear distribution (inset images, compare AR distribution against that of nuclei marked red by expression of an mCherry-based nuclear marker [20]). **B,** CFP and **C,** FRET channel fluorescence from the same field shows the difficulty of detecting those fluorescence levels against high backgrounds that originate with the media only. The images in all three channels are scaled identically for equivalent display.

Fig. 2. Non-uniform backgrounds

A, Representative image of the bright Media Only fluorescence is scaled from minimal pixel intensity (black) to maximum pixel intensity (white). The pink diagonal line shows the trace along which **B,** the intensity at each pixel is much lower at the margins of the image than in the center of the Media Only images, particularly for the Donor (CFP) and FRET channels, although the Acceptor (YFP) channel also shows a similar non-uniformity at a lower intensity level. noise, diagonal traces for Donor, Acceptor and FRET channel images captured in the absence of media or plate.

Fig. 3. Simplistic background correction

A, FRET image and **B,** diagonal pixel intensity trace (dark gray points) across that same image. Pixels along the margins of the image have lower intensities than backgrounds in the middle. The light gray points represent a diagonal trace in the FRET channel across a Media Only background image averaged from many different wells. That Media Only trace fortuitously is of a similar intensity to the background regions in the trace of the experimental image in (A). Simple subtraction of that background image from the experimental image (black points) provides a background subtracted image. **C,** Since backgrounds vary from well to well, similar simplistic background subtractions against other

background images skews the image intensities so that different fluorescent values are obtained. This shows the inherent errors that prevent the use of the simple background subtraction method (section 3.4).

Fig. 4. Masks of non-cellular areas for normalizing the background image to the cellular image **A**, Media Only and **B**, cell-containing image scaled identically and pseudocolored to better accentuate the similarities in background patterns throughout the images. **C**, Automated image segmentation defines objects within the experimental image that are **D**, dilated to create a mask (white area) of the areas where no cells are present. The difference in the intensity between the experimental image and the background image defines a ‘uniform background adjustment’ (section 3.5). It also defines a proportional background adjustment (section 4.1) that provides the most optimal background subtraction protocol.

Fig. 5. Differences in background intensity are proportional to the gradation in intensity over the image

A, Two background images of slightly different average intensities were compared along the diagonal across the image. **B,** The difference in intensity between those two diagonals was less at the image margins than in the center. **C,** Dividing the difference in intensity (B) by the full curves (A) showed the differences in that background between two images to be proportional to the vignetting of the parental images. This proportional characteristic forms the basis for accurate background subtraction (section 4.1).

Fig. 6. Excellent background subtraction through proportional background adjustment
A, Acceptor (YFP) image and diagonal trace (lime line) compared to the diagonal trace from the Media Only control image (gray line). Black line, diagonal trace following background subtraction using the proportional adjustment protocol (section 4.1). Similar success was achieved with the more challenging **B**, Donor (CFP) and **C**, FRET images of the same field.

Fig. 7. Noise determination

The y-intercept of a line drawn between image intensities (y-axis) when the same field is captured over two different exposure times (x-axis) defines the basal 'noise' component of the intensities not affected by exposure time. This noise determination (section 4.2) is required to accurately determine the proportional adjustment (section 4.1) necessary for accurate background subtraction.

Fig. 8. Reproducible measurements upon proportional background adjustment

A, YFP fluorescence intensities in cell nuclei following one day of treatment with 10^{-6} M of the following steroids, Aldo: Aldosterone, T: Testosterone, DHT: Dihydrotestosterone, A-diol: Androstenediol, A-dione: Androstenedione, Androst: Androsterone, DHEA: Dehydroepiandrosterone. **B**, Excellent reproducibility in the efficiency of energy transfer calculated for the same cells following background correction with the proportional adjustment protocol. The data shown represent the mean $\pm$ sd of the field averages for all measurements and indicate the excellent well-to-well reproducibility of the measurements. **C**, Z'-factor scores confirm excellent well-to-well reproducibility in the FRET

measurements for most steroids relative to the control steroid (aldosterone). **D**, The proportion of cells retained for measurement after application of the stringent quality control measures (section 5.4) indicates that fewer cells were accurately measured as AR levels in the nucleus became progressively diminished.

Table 1

Signal noise values determined by exposure in the absence of any object ('in air') or calculated (section 4.2) in the presence of plates, media and cells.

Channel	Integration Time (ms)	Noise in air	Calculated Noise
Acceptor	150	86.2 $\pm$ 1.7	96.7 $\pm$ 4.4
Donor	300	89.2 $\pm$ 1.8	84.2 $\pm$ 1.1
FRET	300	88.8 $\pm$ 1.8	85.5 $\pm$ 6.5