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ORIGINAL ARTICLE

# Fluorescence Observation of Single-Cell cAMP Signaling by G Protein-Coupled Receptors

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## Abstract

We present complementary flow cytometric and microscopic imaging methods, both utilizing a membrane-targeted cAMP sensor protein ICUE3, to examine hormone-dependent signaling by the luteinizing hormone (LH) receptor in individual cells. This receptor, a seven transmembrane domain protein belonging to the GPCR family, signals by activating adenylate cyclase to increase cAMP levels. The ICUE3 sensor protein exhibits fluorescence energy transfer between its CFP and YFP moieties and the ratio of CFP emission to YFP sensitized emission (YFPSE) increases with cAMP concentration. We used multichannel flow cytometry to compare CFP emission and YFPSE from each cell and hence measure that cell's cAMP level. This technique measured changes in cAMP levels in CHO cells expressing LH receptors and stimulated by forskolin or the hormone human chorionic gonadotropin (hCG) and showed that significant cell-to-cell variations exist in such cAMP responses. Because LH receptor behavior may reflect receptor expression levels, we developed a procedure to measure numbers of particular fluorescent cell proteins from measurements of MESF bead standards for slightly different fluorophores. We find that basal cAMP levels increase substantially in cells expressing high numbers mCherry-LH receptors per cell. This suggests activation through increased inter-receptor interactions at high concentrations. We then explored a microscope-based method for single cell measurements so that responses could be correlated with specific cell morphology and with time after treatments. This showed that cell responses to hCG are fully-developed after ~100 s. Taken together, these results demonstrate the utility of fluorescence methods in exploring cAMP signaling in individual cells.

**Keywords** Fluorescence · Cytometry · Microscopy · ICUE · Beads · CFP · YFP · Single cell · G protein coupled · Receptor · Signaling

## Introduction

The LH receptor is a seven-transmembrane domain receptor in the G protein-coupled receptors (GPCR) superfamily [1]. Signal transduction by LH receptors in both males and females is important for successful reproduction. Loss-of-function LH receptor mutations cause a number of diseases including Leydig cell hypoplasia [2]. Familial male-limited precocious puberty (FMPP) is caused by a genetic mutation in the LH receptor resulting in a constitutively-active LH receptor which causes early puberty in males who have high levels of testosterone and low levels of gonadotropin [3]. It has been shown that specific LH receptors can be detected in human endometrial cancer where their expression levels are related to the cancer grade [4]. Activation of the LH receptor by binding human chorionic gonadotropin (hCG) leads to an increase in intracellular levels of adenosine 3′,5′-cyclic monophosphate (cAMP). Of specific interest is whether high levels of LH receptor expression are associated with reduced cell response to ligands that function through the cAMP second messenger. Reduced cAMP response has been previously observed in cells expressing constitutively active LH receptors

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where adenylate cyclase is maximally active in the absence of ligand [5].

To examine effects of expression levels of LH receptor and of a constitutively active construct of LH receptor yoked to hCG (yolked hCG-LH receptor) [6] on intracellular cAMP responses to hormone, both flow cytometric and microscopic imaging methods were used. These measurements were made possible by a fluorescence resonance energy transfer (FRET)-based sensor for intracellular levels of cAMP. The cAMP sensor is based on Epac, an exchange protein directly activated by cAMP. In the Epac1-cAMP sensor, the cAMP binding domain of the Epac1 protein (Epac1 157–316) is anchored by an N-terminal CFP and a C-terminal YFP. Epac2-cAMP sensors use the cAMP binding domain B from Epac2 (Epac2B 284–443) instead. In the absence of cAMP, Epac maintains a folded confirmation with the CFP and YFP moieties in close proximity. Thus much of the fluorescence excitation energy acquired upon deep blue (e.g. 405 nm) excitation of CFP is transferred to YFP and appears as sensitized emission (SE) from the YFP (YFPSE). Binding of cAMP to Epac induces an unfolding of the molecule, increases the CFP-YFP distance, reduces FRET from CFP to YFP, allows more blue excitation energy to appear as CFP fluorescence and so increases the ratio of CFP intensity to YFPSE (reviewed in [7]). cAMP elevating agents such as forskolin increase cAMP, induce ICUE3 conformational changes and result in increased CFP/YFPSE emission ratios. Zhang and coworkers have developed several Epac-based reporters called ICUE probes by using either the full length Epac1 or truncated Epac2 sandwiched between ECFP and a citrine YFP variant [8]. To construct ICUE3, citrine was replaced with a circularly permuted YFP, cpVenus L194, to form the cAMP reporter [9]. Circular permutation introduces new N and C termini and improves the dynamic range by altering the relative orientation of fluorescent proteins.

## Experimental Methods

### Materials

Chinese Hamster Ovary (CHO) cells were purchased from American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle medium (DMEM) was purchased from Corning Cellgro (Manassas, VA). Penicilin/streptomycin and L-glutamine solution were purchased from Gemini Bio-Produucts (West Sacramento, CA). FBS was purchased from Atlas Biologicals (Fort Collins, CO). 100 x MEM non-essential amino acids, bovine albumin, cyclic adenosine monophosphate (cAMP), saponin and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO). Human chorionic gonadotropin (hCG) was purchased from Fitzgerald Industries (Acton, MA). Forskolin (Fsk) was purchased from Enzo Life Sciences (New York, NY). Lipofectamine 3000 and OPTI-MEM reduced serum medium were purchased from Life Technologies (Carlsbad, CA). Quantum R-PE and FITC MESF (molecules of equivalent soluble fluorophore) beads were purchased from Bangs Laboratories (Fishers, IN). Glass bottom cell culture dishes with 35 mm overall diameter and 14 mm diameter, 0.17 mm thick optical glass bottoms were purchased from In Vitro Scientific (Sunnyvale, CA). Filters for fluorescence microscopy were purchased from Chroma Technology (Bellows Falls, VT).

### Cell Culture and Transfection

Chinese Hamster Ovary (CHO) cells were grown in a 25cm$^2$ culture flask in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin and 1% 1x MEM non-essential amino acid solution. All cells were maintained in 5% $CO_2$ at 37 °C in a humidified environment. Before transfection, cells were incubated with 5 mM EDTA for 5 min and 1.5 mL cells were plated in a 35 mm glass bottom Petri dish. Cells grew to approximately 80%–90% confluence in about 2 days.

CHO cells were transiently transfected with 0.4 μg of ICUE3 plasmid, a membrane-targeted cAMP reporter [9] kindly provided by Dr. Jin Zhang (Johns Hopkins University, Baltimore, USA) using Lipofectamine 3000 in accordance with the manufacturer's instructions. Transfection proceeded for at least 12 h. All cells were maintained in a humidified incubator in 5% $CO_2$ at 37 °C. CHO cells were also transiently co-transfected with 0.4 μg ICUE3 and 1.9 μg LH receptor-mCherry using a vector for human LH receptor kindly prepared by Dr. Xiaorong Li at Southwest University, Chongqing, China. We also obtained a constitutively active form of LH receptor from Dr. P. Narayan in which the β and α subunits of hCG are expressed in tandem at the N-terminus of the LH receptor(hCG-β-hCG-α-LH receptor) as a single polypeptide chain and termed the "yolked" hormone-receptor complex (yolked hCG-LH receptor). Co-transfection procedures were similar to those used to transfect CHO cells with ICUE3 alone. After cells were transiently transfected with ICUE3 alone, with ICUE3 and LH receptor-mCherry or with ICUE3 and yolked hCG-LH receptor, medium was aspirated from the Petri dish, cells were washed twice gently and maintained in 1x phosphate buffered saline (PBS) pH 7.4 containing 0.1% BSA. Cells were then imaged using procedures described below.

### Flow Cytometric Measurements of CFP, YFP and mCherry on Individual Cells

Flow cytometry experiments employed a Moflo Astrios EQ flow cytometer located at the University of Colorado Cancer Center [10]. Simultaneous measurement of CFP emission, YFP sensitized emission, YFP direct emission and mCherry emission was made possible by excitation with three non-colinear lasers as shown in Table 1. The table also indicates the bandpass filters used to isolate each fluorescence signal. Forward and 90° scatter at 488 nm were recorded as well. These six detector signals for typically 200,000 individual cells in an individual experiment were recorded by the cytometer in FCS-format files and subsequently transferred to Excel spreadsheets for analysis. While such files were lengthy and somewhat awkward in analysis procedures, the data permitted measuring cAMP hormone responses of each cell and relating these responses to absolute numbers of receptors expressed in that particular cell. The ratio of ICUE3 cyan fluorescence to the corresponding YFPSE provided an easily-evaluated measure of cellular cAMP. Numbers of receptors and ICUE3 molecules on individual cells were evaluated as described below.

### cAMP Determination on Individual Cells via Microscopic Ratio Imaging

Fluorescence images were collected in a Zeiss Axiovert 200 M inverted microscope using a 1.2 NA 63x water objective and an Andor DU-897 EMCCD camera controlled by MetaFluor software. A 10% neutral density filter was used to reduce arc lamp intensity to reduce fluorophore photobleaching. Filters used were as shown in Table 1 plus a 455DRLP dichroic mirror (Chroma) The coverslip-bottomed Petri dish was secured to the microscope to minimize sample movement. Sequences of about 30 images were taken at 30s or 60s intervals. During experiments, data were collected from untreated cells for several minutes before the PBS solution was removed and replaced with either 50 μM forskolin or 10 nM hCG in PBS. In some experiments, a standard curve for intracellular cAMP was constructed using cells treated with 0.1 nM - 1000 nM cAMP in PBS, pH 7.4, containing 0.02% saponin. Because the effect of saponin is reversible, saponin remained in solutions containing cAMP during image acquisition. All data were analyzed by Image J software. Fluorescence images were initially corrected by subtracting the field background intensity from the emission intensities of fluorescent cells expressing ICUE3. Emission ratios (CFP/YFPSE) were calculated from corrected CFP emission intensity and corrected YFPSE intensity, i.e. YFP emission resulting from CFP excitation. Individual measurements were typically made on 14–24 cells under each experimental condition.

## Results and Discussion

**Individual Cell cAMP Levels Can Be Measured by Flow Cytometry** CHO cells were transiently transfected with ICUE3 or with ICUE3 and LH receptor-mCherry. The yellow direct fluorescence from membrane-targeted ICUE3 was associated with the plasma membrane as was red fluorescence from LH receptor-mCherry. Changes in ICUE3 fluorescence emission in response to cAMP concentration were examined using flow cytometry. For each cell, the ratio of ICUE3 CFP fluorescence to YFPSE was calculated. To obtain a standard curve relating intracellular cAMP to this ratio, CHO cells expressing ICUE3 were treated with various concentrations of cAMP in 0.02% saponin in PBS. The standard curve obtained is shown in Fig. 1. There was only a small change in the ratio of CFP emission to YFP sensitized emission upon 405 nm excitation in cells treated with 0 μM, 0.1 μM or 1.0 μM cAMP. Larger increases in the CFP to YFPSE ratio were observed for cells treatment with 10 μM or 100 μM cAMP which, as expected, reduced the YFPSE signal. The $EC_{50}$ value of for cAMP was in the range 10 μM to 100 μM which agrees with a previous study using ICUE2 [11].

**cAMP Signal from ICUE3 Sensors Remains Constant over a Broad Range of ICUE3 Expression Levels** The effects of ICU3 expression levels were then evaluated on CHO cells expressing ICUE3 alone or in cells treated with forskolin. Figure 2 shows that increasing levels of ICUE3, indicated as increases in directly excited YFP intensity, cause no change in the average CFP to YFPSE ratio despite a broad range of ICUE3 expression levels. Thus increased numbers of ICUE3 per cell did not alter the ratio of CFP fluorescence to YFPSE under conditions where basal cAMP remained constant. Moreover,

**Table 1** Excitation and emission filters used to measure CFP, YFPSE, YFP and mCherry fluorescence in flow cytometry and imaging microscopy experiments, respectively

| Fluorescence source | Flow cytometry | | Microscopy | | |
|---|---|---|---|---|---|
| | Laser Exc. (nm)[a] | Emission (nm) | Excitation (nm) | Beam splitter (nm) | Emission (nm) |
| CFP | 405 | 448/50 | 436/20 | 455 | 480/40 |
| YFP (SE) | 405 | 525/40 | 436/20 | 455 | 535/30 |
| YFP (direct) | 488 | 525/40 | – | – | – |
| mCherry | 561 | 579/16 | 545/30 | 570 | 610/75 |

[a] For excitation in flow cytometry only the laser wavelength is given. In all other cases filters are specified as "center wavelength/bandwidth"

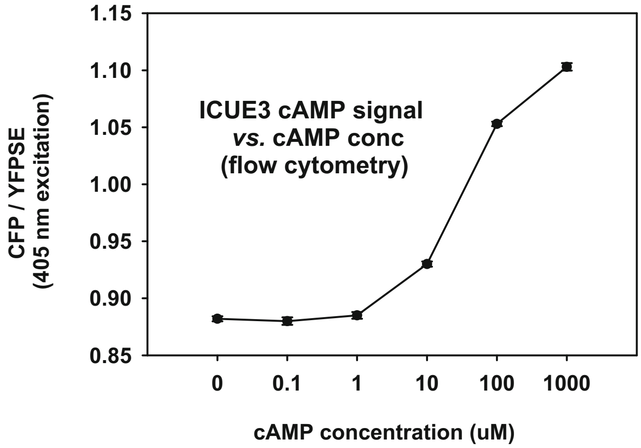



**Fig. 1** Standard curve obtained using flow cytometry showing the relationship between the CFP/YFPSE emission ratio and intracellular cAMP levels. There is a small change in CFP/YFPSE for cells exposed to 1 μM cAMP. Larger increases are seen for cells treated with cAMP concentrations ≥10 μM as indicated by increased CFP/YFPSE emission ratios. Data shown are the mean ± S.D. where the number n of cells examined is greater than 1000

while this ratio varies substantially among individual cells in any population as shown in Fig. 2, the *average* ratio is quite constant for samples exposed to a given hormone concentration. 50 μM forskolin increased, on average, the CFP to YFPSE ratio which, as in untreated cells, was not dependent on ICUE3 expression levels (data not shown).

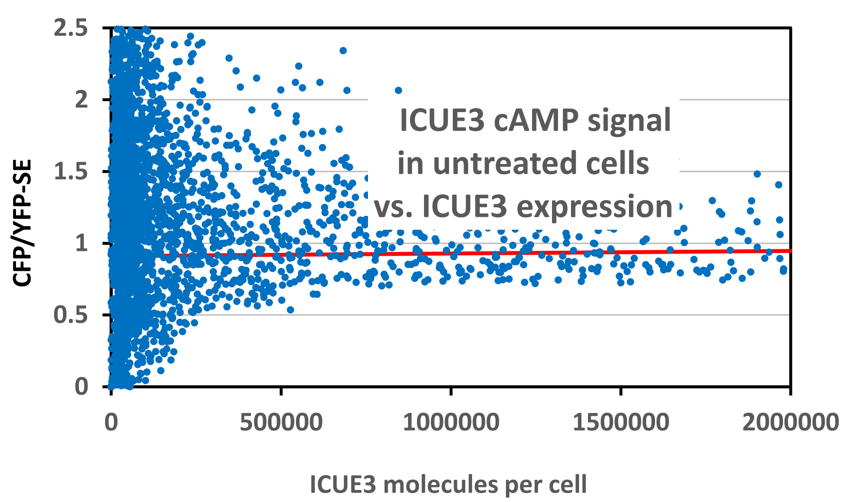
**Absolute Numbers of Fluorescent Proteins per Cell Can Be Estimated Using MESF Bead Standards Involving in a Different Fluorophore** Flow cytometry was also used to examine the relationship between absolute numbers of LH receptors per cell and cAMP signals in cells co-transfected with ICUE 3 and LH receptor-mCherry. Absolute expression numbers for mCherry and the YFP moiety of ICUE3 in cells co-transfected with LH receptor-mCherry and ICUE3 were extracted from flow cytometry data. Availability of bead standards containing known amounts of particular fluorophores is quite limited and no such standards are available for either YFP or mCherry fluorophores. However, bead standards for somewhat similar fluorophores, FITC or R-phycoerythrin (R-PE) are available from Bangs Laboratories. The spectral properties of FITC relative to YFP and of R-PE relative to mCherry were used [12, 13], together with cytometry filter parameters and wavelength variations in detector sensitivity, to relate absolute numbers of FITC and R-PE fluorophores on beads to numbers of YFP and mCherry, respectively, on cells (Fig. 3).

The detected fluorescence signal $R_B$ in photons per second for one dye molecule on a calibration bead observed in the cytometer is obtained by the equation:

$$R_B = A\,\varepsilon_B\left(\lambda_{\max,B}\right)\left(\frac{a_B\left(\lambda_{exc,B}\right)}{a_B\left(\lambda_{\max,B}\right)}\right)\Phi_B\frac{\int_{\lambda_{c,B}-w_B/2}^{\lambda_{c,B}+w_B/2} f_B(\lambda)\,e(\lambda)d\lambda}{\int_0^{\infty} f_B(\lambda)d\lambda} \tag{1}$$

where $\varepsilon_B$ ($\lambda_{max,B}$) is the molar absorptivity of the given bead dye molecule at its maximum absorption wavelength $\lambda_{max,\mathbf{B}}$ and $\Phi_B$ is the fluorescence quantum yield. The ratio $a(\lambda_{exc,B})/\ a(\lambda_{max,\mathbf{B}})$ is the fractional absorbance for the bead dye molecule at the excitation wavelength $\lambda_{exc,B}$ relative to that at $\lambda_{max,B}$ and the center wavelength and bandwidth of the emission filter are $\lambda_{c,B}$ and $w_B$, respectively. The upper integral in Eq. 1 is the integral of the bead fluorophore corrected emission intensity $f_B(\lambda)$ times the detector quantum efficiency $e(\lambda)$ across the bead fluorophore filter bandwidth and the lower integral is the corresponding integral over all wavelengths. The constant A, actually unimportant here, involves the exciting light wavelength λ, the intensity I on the chromophore of exciting light, the efficiency e of delivery of fluorescence photons to the detector and subsequent detection of primary photoelectrons, Avogadro's number $N_A$, Planck's constant h and the speed of light c.

**Fig. 2** The relationship between ICUE3 number (YFP emission with 488 nm excitation) and CFP/YFPSE fluorescence ratio upon 405 nm excitation. The Figure shows basal cAMP levels in cells expressing various levels of ICUE3 as indicated by YFP intensity. Increasing levels of ICUE3 per cell do not alter the ratio of CFP fluorescence to YFP sensitized emission



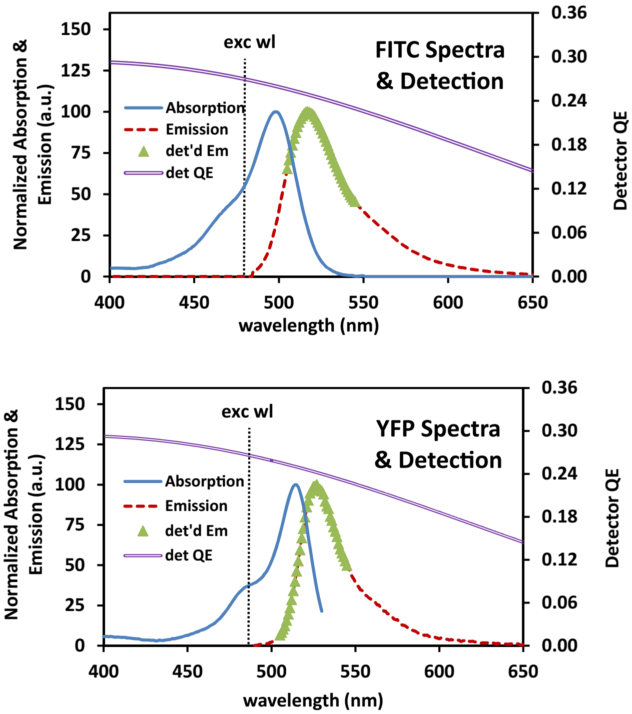



**Fig. 3** Comparison of spectra for FITC (upper panel) and for YFP (lower panel). Each panel shows the indicated chromophore's normalized absorption spectrum (solid blue line), fluorescence emission spectrum (dashed red line) and detector quantum yield (double purple line). The bandpass of the cytometer emission filter is shown as green triangles superimposed on the fluorescence emission spectrum. The excitation laser wavelength, here 488 nm, is indicated by the dotted black line

$$A = \frac{2303\,\lambda I\, e}{N_A h c} \tag{2}$$

The detected fluorescence signal $R_P$ for one fluorescent protein molecule on a cell is given by Eq. 1 but with all subscript "B" changed to "P" to indicate quantities for the fluorescent protein. The ratio $R_B$ / $R_P$ can thus be calculated for a pair of bead and protein fluorophores from photophysical data. Figure 4 shows spectral data from which various required quantities may be obtained.

Cytometric measurements of beads using emission filters appropriate to the target protein provided calibration curves of the Manufacturer's numbers $N_B$ of FITC or R-PE per bead versus measured mean fluorescence intensity $S_B$ of FITC or R-PE per bead. The slope $N_B/S_B$ of such a curve is the number of bead fluorophores per unit bead fluorescence detected under conditions used to measure the protein of interest. Measurements of protein fluorescence $S_P$ were then obtained for cells using the same emission filters. Finally, the number of fluorescent protein molecules $N_P$ expressed on each cell is given by

$$N_p = \left(\frac{N_B}{S_B}\right) * \left(\frac{R_B}{R_p}\right) * S_P \tag{3}$$

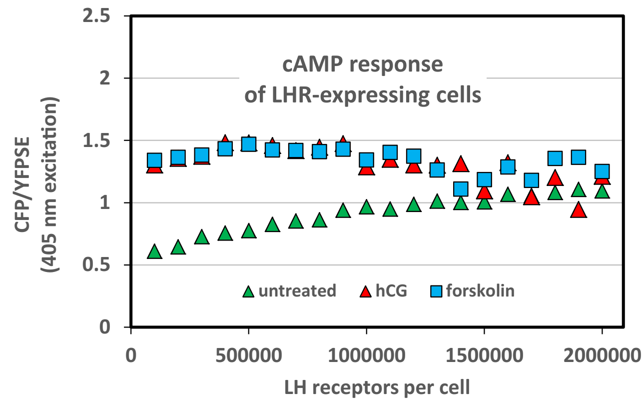



**Fig. 4** CFP/YFPSE emission ratios for CHO cells co-expressing ICUE3 and hLHR-mCherry and where cells are either untreated (green triangles) or treated with 50 μM forskolin (blue squares) or 100 nM hCG (red triangles). The CYP/YFPSE ratio is shown for numbers of mCherry LH receptors up to 2,000,000 per cell. The increase in the CFP/YFPSE emission ratio with receptor expression likely reflects receptor activation from increasing inter-receptor contacts at high expression levels

As an example, for FITC, peak molar absorptivity is 75,800 L $mol^{-1}$ $cm^{-1}$ at 498 nm, the fraction of that maximum molar absorptivity is 0.77 at the excitation wavelength of 488 nm, the fluorescence quantum yield is 0.48, the (upper) integral over the filter bandwidth is 778 nm and the total emission (lower) integral is 5010 nm. Equation 1 then gives $R_b$ as A*4373 $s^{-1}$. For YFP, peak molar absorptivity is 84,000 L $mol^{-1}$ $cm^{-1}$ at 514 nm, the fraction of that maximum molar absorptivity is 0.38 at the excitation wavelength of 488 nm, the fluorescence quantum yield is 0.61, the (upper) integral over the filter bandwidth is 626 nm and the total emission (lower) integral is 3808 nm. From Eq. 1, $R_P$ is A*3212 $s^{-1}$. The ratio $R_B$ /$R_P$ is then 1.36 and thus the number of YFP per cell is 1.36 times the number that would be inferred from FITC bead standards assuming the cells bore FITC-tagged molecules.

**Individual Cells' cAMP Responses Decrease for Large Numbers of Expressed Receptors** The cAMP signal obtained from cells expressing a range of LH receptor per cell is shown in Fig. 4. In the absence of treatment with either hCG or forskolin, the ratio of the CFP to YFP signal varied with numbers of expressed receptors. Low numbers of LH receptor-mCherry per cell, 100,000 receptors/cell, were associated with low CFP/YFPSE emission ratios indicating that there was significant FRET occurring in the ICUE3 probe and that levels of cAMP per cell were comparatively low. As receptor numbers increased above 100,000/cell, the CFP/YFPSE emission ratio increased significantly indicating elevated cAMP levels. This suggests activation through increased inter-receptor interactions at high concentrations. As very high expression levels approach 2,000,000 receptors per cell, cAMP levels approach those exhibited by hCG- or forskolin-induced stimulated cells.

This suggests that such trans-activation can become quite efficient. We note that the CFP/YFPSE emission ratios here are somewhat lower than those shown in Fig. 1 which refers to cells expressing ICUE3 only. It is possible that cells of Fig. 4 co-transfected for both ICUE and LHR-mCh simply exhibit lower basal levels of cAMP than do cells expressing ICUE alone.

When CHO cells with fewer than 500,000 LH receptor/cell were treated with 100 nM hCG, there was an increase in CFP/YFPSE emission ratio with a maximum change of >2-fold compared to untreated cells. This result was consistent with hCG effects on LH receptor-mediated activation of adenylate cyclase and increased intracellular levels of cAMP [14]. Interestingly, cells expressing over 1,000,000 LH receptors per cell were less responsive to hCG. This pattern of responsiveness was also observed for treatment with forskolin.

One explanation for these observations is that LH receptors, when expressed at numbers greater than 500,000, become both aggregated and constitutively active. For some membrane proteins, formation of high order oligomers and aggregates occurs more readily in crowded environments [15]. These constitutively active receptors have reduced or no capacity for hormone or forskolin response. These results are also consistent with the biological state in which native receptors, expressed at relatively low numbers, are responsive to either luteinizing hormone or, for human cells, human chorionic gonadotropin. As a point of reference, the number of native LH receptors in, for example, rat Leydig cells, is dependent on the stage of cell development and are generally expressed at numbers less than 15,000 receptors per cells [16].

### ICUE3 Permits cAMP Measurements by Imaging Microscopy

Flow cytometry allows examination of large numbers of cells and evaluation of effects of LH receptor-mCherry numbers on cAMP signals. However, as shown in Fig. 4, there was overlap between cAMP levels on a per cell basis for untreated, hCG-treated and forskolin-treated cells when LH receptor numbers were greater than 1,500,000 receptors per cell. This made it difficult to assess changes in cAMP levels with cell treatments. To overcome this problem, it was useful to examine individual cells where treatment effects on a single cell could be monitored directly. Initial studies evaluating effects of cAMP on the ICUE3 signal are shown in Fig. 5. Increasing concentrations of cAMP result in an increase in the ratio of CFP to YFPSE. This indicates that ICUE3 is responding to increased cAMP with a decrease in the FRET signal. These

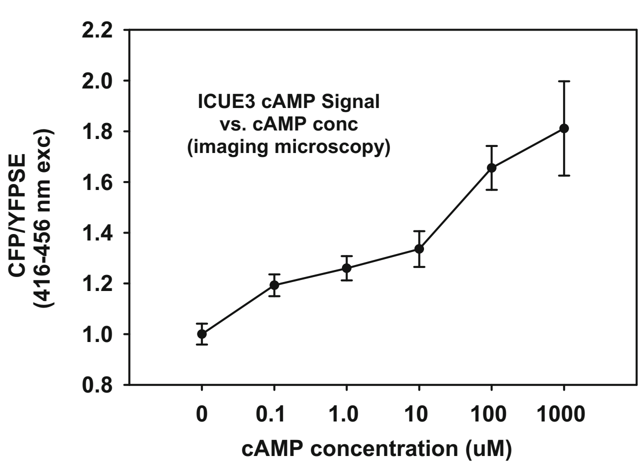
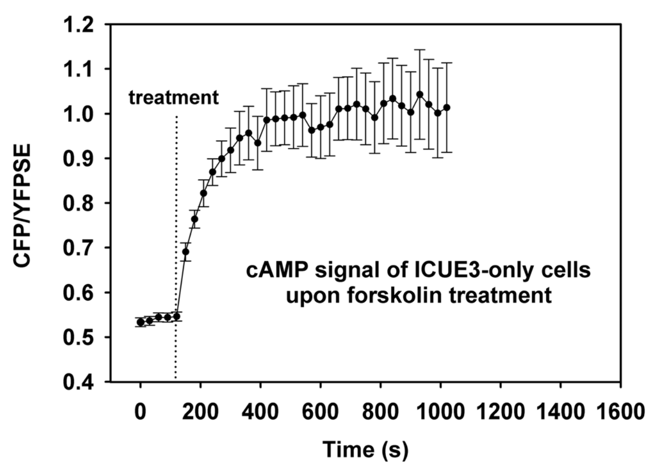



**Fig. 5** CFP/YFPSE emission ratio of CHO cells expressing ICUE3 treated by 0.1 μM, 1.0 μM, 10 μM, 100 μM and 1000 μM cAMP obtained using a microscope-based system with CFP excitation at 436 nm. Data shown are the mean ± S.D. for $n = 4$ experiments



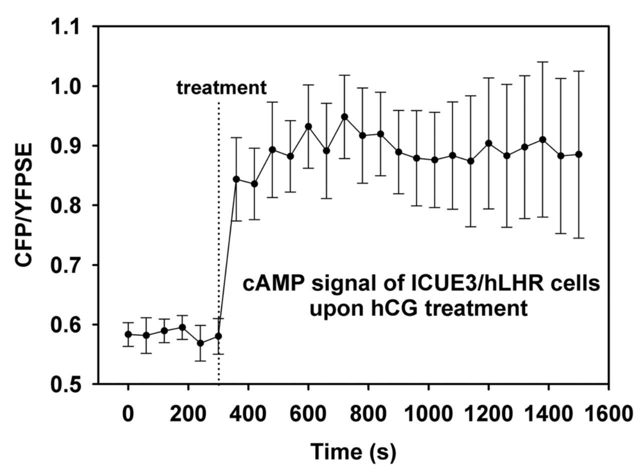




**Fig. 6** (Upper panel) Effect of 50 μM forskolin on the CFP/YFPSE emission ratio in cells expressing ICUE3 alone. CHO cells that were attached to a glass coverslip that had been connected to an opening in a petri dish. Forskolin was added at the indicated time by quickly exchanging medium in the Petri dish. Images were acquired every 30 s before and after exchange of cell medium. Data shown are the mean ± SD ($n = 6$). (Lower panel) Effect of 100 nM hCG on CHO cells added at the time indicated to cells co-expressing ICUE3 and hLHR-mCherry. hCG treatment produced a rapid increase in intracellular cAMP as indicated by increase in the ratio of CFP/YFP emission. Images were taken at 60 s intervals. Data shown are the mean ± S.D. for 5 measurements for each point

data show the CFP/YFP-SE emission ratio for cells as determined by *imaging microscopy* of individual cells. By contrast, Fig. 1 reflects measurements using *flow cytometry*. The filters and excitation wavelength used in the microscope are different from those used in the cytometer. As a consequence, a given individual cell will necessarily yield somewhat values of CFP/YFP-SE when examined using the two techniques.

**cAMP Responses of Individual Cells to Hormone Can Be Followed Microscopically** Representative data showing the effects of treating a CHO cell with forskolin treatment on a CHO cell expressing ICUE3 alone (Fig. 6, upper panel) and on hCG treatment of a CHO cell expressing ICUE3 and LH receptor-mCherry (Fig. 6, lower panel). The ICUE3-only cells respond to 50 nM forskolin, indicating activation of adenylate cyclase. Behavior of the cell expressing ICUE3 and LH receptor-mCherry and treated with 100 nM hCG demonstrates that adenylate cyclase is available and can be activated by hormone. This figure shows time-course measurements on individual cells taken at random from a transfected cell population. Hence the actual numbers of LH receptors on the particular cells examined cannot be known. However, flow cytometry suggests that our LHR-mCh transfection protocol yields a most-probable receptor number of about 900,000 receptors per cell.

**Individual Cells Transfected with Yolked hCG-LH Receptor Demonstrate Constitutive Activity Not Enhanced by Forskolin or Hormone** The hormone-receptor complex comprising the covalently yoked hCG-LH receptor is constitutively active, presumably because of the fixed proximity of the hCG subunits to their LH receptor binding site [17]. The microscope-based method was used to examine cAMP levels in single cells expressing constitutively-active yolked hCG-LH receptor. As shown in Table 2, cells were transfected with yolked hCG-LH receptor with or without ICUE3. In some experiments, mCherry was inserted in the vector for yolked hCG-LH receptor to verify expression of the vector's gene products. Cells were then treated with either 100 nM hCG or 50 mM forskolin after first obtaining baseline values for the CFP/YFPSE emission ratio. The CFP/YFPSE emission ratio before and after treatment were calculated for each individual cells. As predicted from flow cytometry results (Fig. 2), there was considerable cell to cell variation in initial CFP/YFPSE emission ratios before treatments regardless of whether cells expressed ICUE3 alone or together with yolked hCG-LH receptor. For cells expressing ICUE3 alone, 50 mM forskolin increased cAMP expression while 100 nM hCG treatment had no effect. When both yolked hCG-LH receptor and ICUE3 were co-transfected into CHO cells, the CFP/YFPSE emission ratio remained generally unchanged which suggested that cells were already maximally producing cAMP. Control cell populations that lacked ICUE3 showed no effect of either forskolin or hCG. The absence of a response to either forskolin or hCG in cells transfected with yolked hCG-LH receptor was consistent with previously published studies demonstrating constitutive activity of this receptor in cell lines [6] or development of a phenotype characteristic of precocious puberty in rats expressing the receptor in vivo [18]. In our hands, the absence of CHO cell response occurs despite the presence of functional adenylate cyclase in CHO cells. This is shown in cells lacking the receptor where there is a 1.35-fold change in of ratio of CFP to YFPSE with forskolin. The magnitude of this change was similar to the ratio of CFP to YFPSE, 0.30 and 0.45, for untreated cells and cells treated with forskolin, respectively, in cytometry studies.

**Table 2** Single cell imaging of ICUE3 CFP and YFPSE fluorescence to evaluate effects of cell treatment with either 50 μM forskolin or 100 nM hCG on intracellular cAMP levels

| Vectors used for cell transfection | | | Treatment | CFP/YFPSE emission ratio (reflecting cAMP level)[a] | | Treatment effect on cAMP levels (emission ratio after / emission ratio before) |
|---|---|---|---|---|---|---|
| yolked-hCG-LH receptor | ICUE3 | mCherry | | Before treatment | After treatment | |
| – | + | – | 100 nM hCG | 1.05 ± 0.008 | 1.06 ± 0.008 | 1.03 |
| – | + | – | 50 μM forskolin | 0.84 ± 0.008 | 1.11 ± 0.050 | 1.35 |
| + | + | – | 50 μM forskolin | 0.89 ± 0.004 | 0.94 ± 0.009 | 1.06 |
| + | + | – | 100 nM hCG | 1.22 ± 0.010 | 1.30 ± 0.040 | 1.11 |
| + | – | + | 100 nM hCG | 0.52 ± 0.010 | 0.56 ± 0.008 | 1.09 |
| + | – | + | 50 μM forskolin | 0.40 ± 0.001 | 0.46 ± 0.003 | 1.04 |
| – | – | + | 100 nM hCG | 0.83 ± 0.008 | 0.90 ± 0.005 | 1.09 |
| – | – | + | 50 μM forskolin | 0.64 ± 0.004 | 0.70 ± 0.004 | 1.07 |

[a] The results for values before treatment and after treatment are the mean and SEM for 14–24 measurements on individual cells

## Conclusions

Together, these results demonstrate major advantages to flow cytometry and single cell imaging methods for evaluating intracellular cAMP levels. Flow cytometric methods can evaluate cAMP levels in large numbers of cells and can be used to estimate cAMP levels on a per-cell basis within a cell population. Single cell imaging measurements are useful in evaluating time-dependent changes in cAMP in response to, for example, hormone treatment, and permit measurements on individual cells selected on the basis of morphology. In comparison, there are large cell-to-cell variations in basal cAMP levels so that small changes in cAMP, detected in single cell imaging, will be less apparent in flow cytometry studies of large cell populations.

Biologically, these results suggest that LH receptors activate adenylate cyclase via a hormone-mediated mechanism most effective at receptor numbers of <500,000 per cell. This signaling can be adversely affected by overexpression of the receptor. LH receptors expressed at numbers >500,000 receptors/cell signal, at least in part, through transient ligand-induced aggregation and translocation to lipid rafts. Higher receptor surface densities may drive permanent aggregation simply through concentration effects and lead to a degree of constitutive unresponsiveness.

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