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4-Hydroxytamoxifen Differentially Exerts Estrogenic and Antiestrogenic Effects on Discrete Subpopulations of Human Breast Cancer Cells

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Functional heterogeneity within populations of breast cancer cells contribute to the seemingly paradoxical effects of antiestrogens and the development of antiestrogen "resistance." Our objectives were to determine the degree to which T-47D cells may respond inappropriately (positively) to the antiestrogen 4-hydroxytamoxifen (HOT) alone, and whether all cells that respond to the stimulatory effects of estradiol-17β (E_2) are inhibited by the addition of HOT. Single, living T-47D cells were transfected by microinjection with an estrogen response element (ERE)-driven luciferase reporter plasmid. Transfected cells were then treated with medium alone, HOT, E_2 or a combination thereof on consecutive days, exposed to the substrate luciferin and subjected to quantification of photonic emissions reflective of ERE-stimulated activity. This analysis revealed a subpopulation of cells that exhibited increased ERE-driven photonic activity in response to HOT. In companion studies, E_2-stimulated ERE activity was reversed (on average) with HOT addition. However, analysis of individual cells revealed that although HOT reduced photonic activity in the majority (89.2%) of E_2-responsive cells, there was a small subset (10.8% of the population) that was stimulated by E_2 + HOT cotreatment. Our data support the hypothesis that these cells possess an intrinsic "resistance" to antiestrogenic agents, and that this could contribute to the remodeling of tumor cell populations toward a "resistant" phenotype.

Key Words: Breast cancer; tamoxifen; estrogen; 4-hydroxytamoxifen; gene expression.

Introduction

The antiestrogen tamoxifen (TAM) has been used to treat estrogen-responsive breast cancers for more than two decades. Unfortunately, many of the patients who exhibit an initial appropriate response to the drug (tumor regression or postsurgical nonrecurrence) eventually become refractory to it. This loss of responsiveness, called TAM resistance, has been studied extensively *(1–3)*. Nevertheless, the mechanisms governing its development have yet to be elucidated completely, and multiple theories (many of which are not mutually exclusive) have been put forth to explain this phenomenon. A thread common to most of these proposals is that TAM resistance is the result of some alteration in estrogen receptor (ER) signal transduction, because TAM and its metabolite (4-hydroxytamoxifen, [HOT]) are believed to act through this pathway.

The search for the mechanisms underlying acquisition of the "resistant" phenotype has been complicated by the fact that human breast cancers as well as breast cancer cell lines exhibit varying degrees of morphologic and functional heterogeneity *(1,4–7)*. Put simply, not all tumor cells from a single source are alike; some respond very differently (or not at all) to the same stimulus. This innate heterogeneity has led to the intriguing suggestion that TAM resistance is not "acquired" in the traditional sense but, rather, is the consequence of positive selection of cells (present prior to therapeutic intervention) on which TAM acts paradoxically as an agonist or has no effect at all *(1)*. Although there is evidence consistent with this theory, it invariably derives from studies in which averaged responses were measured on entire populations (cultures) of cells. Clearly, a testing of the hypothesis of differential cellular responsiveness to TAM would be greatly facilitated by the resolving power of single cell analysis. This notion is reinforced by previous studies that have characterized the remodeling of breast cancer cell populations by TAM using flow cytometric analysis *(8)* and other techniques.

The system our laboratory has developed to enable study of single-cell responsiveness to estrogenic agents involves transfecting individual, living breast cancer cells with an

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estrogen-responsive luciferase reporter followed by quantitative evaluations of estrogen- and antiestrogen-mediated luciferase activity (in the form of photonic emissions) using low-light monitoring cameras. Through this technical approach, the ability of individual cells within a population to respond differentially to estrogenic and antiestrogenic agents is being examined. In the present investigation, we determined in single, living T-47D breast cancer cells the degree to which individual cells may respond to HOT treatment alone, and whether all cells within a population that respond to the stimulatory effects of estrogen are inhibited by the addition of the antiestrogen HOT.

Results

HOT Alone Stimulates Estrogen Response Element–Driven Luciferase Activity in Single, Living T-47D Cells

Photonic emissions indicative of specific estrogen response element (ERE)-driven reporter activity from individual, transfected T-47D cells were quantified as described previously *(9)* and as depicted in Fig. 1 (see Materials and Methods). As a first step, we had to establish the basal (nonstimulated) level of ERE-driven reporter activity generated by transfected cells in our system. To accomplish this, we microinjected T-47D cells with the MMTV-ERE-LUC plasmid, maintained them in medium alone (no estradiol-17β [E_2]) supplemented with charcoal-stripped fetal bovine serum (cs-FBS), and imaged them on consecutive days. Under these estrogen-depleted conditions, the vast majority of cells did not produce a photonic signal that could be distinguished from background levels. This was established clearly after examination of cells on 20 gridded cover slips generated in eight independent experiments. However, a very weak but quantifiable signal was detected in a small minority of cells. Indeed, only 19 cells (as shown in Fig. 2A) out of more than 300 cells examined exhibited any luciferase activity. These data demonstrate the strict requirement of estrogen for activation of the MMTV-ERE-LUC reporter in T-47D cells.

Parallel experiments aimed at examining the ability of E_2 alone or HOT alone to stimulate ERE-driven reporter activity were also conducted. In these experiments, transfected cells were exposed for 24 h to medium alone and then imaged on d 1. This baseline measurement was followed by treatment of the cells with HOT (1 μ*M*) (Fig. 2B) or E_2 (1 n*M*) (Fig. 2C) for 24 h, after which cells were subjected again to photonic imaging (d 2). As already described, cells exposed to E_2-depleted medium alone for 24 h exhibited either a weak photonic signal or none at all. As might have been anticipated, exposure of cells to E_2 resulted in the detection of increasing reporter activity in the vast majority of cells studied (98.5%) (Fig. 2C). On average, E_2 treatment evoked an 18.7-fold increase ($p < 0.05$) over basal values of specific photonic emissions. Interestingly, treatment with HOT also induced an unequivocal increase ($p < 0.05$) of ERE-driven reporter activity, but it did so in only a discrete minority subset of cells examined (25.5%) (Fig. 2B). In those responsive cells, HOT enhanced ($p <$

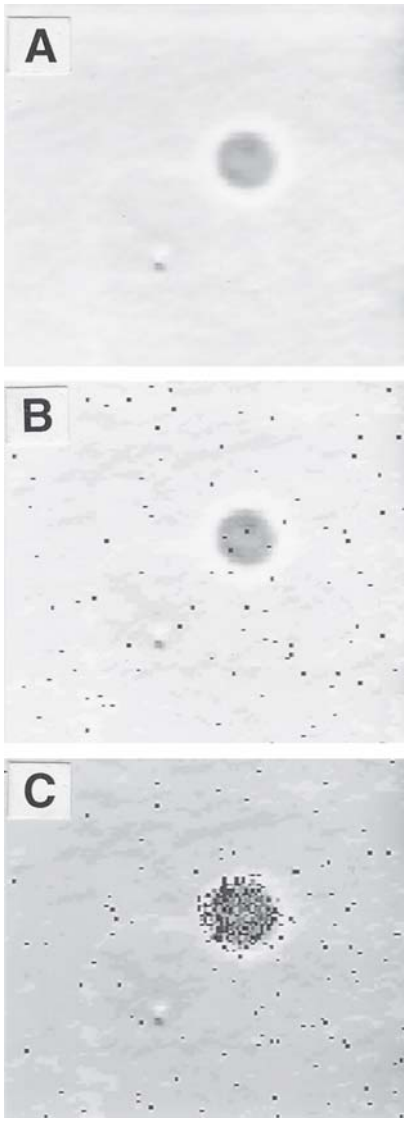

Fig. 1. (**A**) T-47D cell (bright-field-captured image; ×400); (**B**) basal and (**C**) estrogen-stimulated MMTV-ERE-LUC activity in a single, living breast cancer cell. The T-47D cell in (A) was transfected by microinjection with the MMTV-ERE-LUC reporter construct and incubated for 24 h in medium alone containing 10% cs-FBS. Then, the transfected cell was exposed to luciferin and imaged for 10 min to detect photonic activity (B). Note that the cell depicted in (B) shows no cell-specific photonic activity under these basal (E_2-free) conditions. Following imaging on d 1, the cell was treated with 1 n*M* E_2 and incubated an additional 24 h prior to imaging (10 min) on d 2 (C). Shown in (C) are amplified photonic signals superimposed on the bright-field image (A) of the cell from which these signals derived. Differences in color here are indicative of rate of photonic emissions consistent with the ascending color scale on the right side of (C). These images demonstrate not only the sensitivity of the MMTV-ERE-LUC plasmid to stimulation with E_2 in a T-47D cell, but also the resolving power of this photon capture system for localizing photonic signals to an individual, living cell.

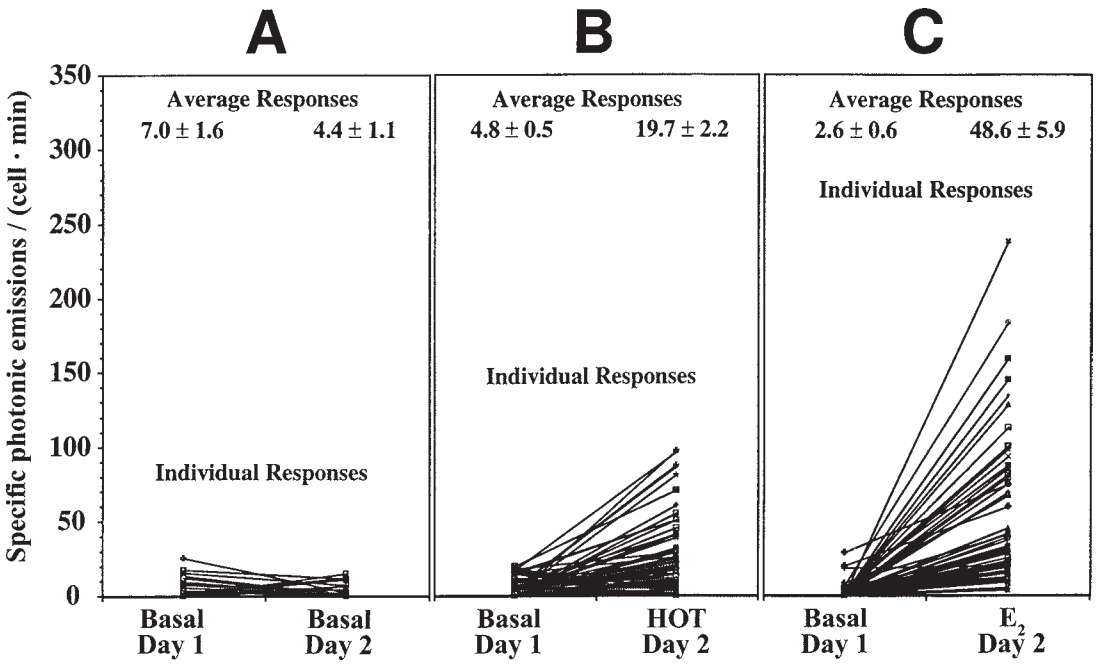

Fig. 2. Effects of estrogen (E_2) and antiestrogen (HOT) on ERE-driven luciferase activity in T-47D cells. All cells in each group (**A–C**) were maintained in control medium and subjected to photonic imaging (d 1) 24 h after transfection with the MMTV-ERE-LUC plasmid. They were then cultured for an additional 24 h in (**A**) control medium, (**B**) HOT (1 μ*M*), or (**C**) E_2 (1 n*M*), after which they were subjected again to photonic imaging (d 2). As discussed in detail under Results, luciferase activities in all groups were extremely low or undetectable under basal conditions. Treatment with E_2 increased the rate of photonic emissions in the vast majority of transfected cells studied (n = 65), (C). Interestingly, HOT mimicked the stimulatory effects of E_2 in a small but significant subset of T-47D cells (n = 106). For this and Fig. 3, points on d 1 and 2 connected by a line represent the specific photonic emissions detected for the same cell on consecutive days. Average responses are mean ± SEM.

0.05) the rate of photonic emissions 4.1-fold relative to basal values.

Differential Antagonism of ERE-Regulated Gene Expression by HOT

Having established that HOT could act as an E_2 agonist in a minority subset of T-47D cells, we next determined whether the vast majority of cells that responded to E_2 might respond differentially to cotreatment with HOT (Fig. 3). We treated transfected cells with E_2 (1 n*M*) and then imaged the cells 24 h later (d 1). Immediately thereafter, some cells were exposed for a subsequent 24 h to E_2 again whereas others were treated with E_2 + HOT (1 μ*M*) and imaging was repeated (d 2). Consistent with our previous finding *(9)*, continuous E_2 treatment significantly elevated ($p < 0.05$) ERE-driven luciferase activity on both days (Fig. 3A). Although the average level of elevated photonic emissions in cells treated continuously with E_2 did not differ ($p > 0.10$) from one day to the next, individual cells clearly fluctuated from day to day in their relative levels of photonic emissions (46.3% increasing and 53.7% decreasing from d 1 to d 2) (Fig. 3A), thereby demonstrating with our single cell model a dynamic instability of gene expression that could not be detected by monitoring these responses at the population level. While this heterogeneity in gene expression among single cells is not clearly understood, it is thought that following the addition of E_2 there may be differences among cells in hormone receptor activity (i.e., upregulation and downregulation) or in their progression through the cell cycle.

In examining the actions of HOT on ERE-mediated luciferase activity subsequent to E_2 stimulation (Fig. 3B), we found that the superimposition of HOT suppressed E_2 activation of reporter activity in most of the cells studied (89.2%) (Fig. 3B, main panel). However, the antiestrogen had no effect or actually increased ERE-driven luciferase activity in others (10.8%) (Fig. 3B, inset). Notably, this differential effect of HOT was masked when only the population (i.e., average) responses were considered. This was evidenced by the 4.3-fold decrease ($p < 0.05$) in photonic emissions observed when all cells (Fig. 3B, main panel and inset) were included in a collective analysis (mean ± SEM: d 1 = 37.3 ± 4.6 vs d 2 = 8.7 ± 2.0 specific photonic emissions/min). On further examination of the individual cells that responded positively to the addition of HOT in the presence of E_2, we found that these cells actually increased ($p < 0.05$) twofold in ERE-driven photonic emissions over E_2 treatment alone from the previous day (Fig. 3B, inset). Moreover, E_2 + HOT-stimulated cells (Fig. 3B, inset; d 2) were similar ($p > 0.10$) in their mean levels of photonic

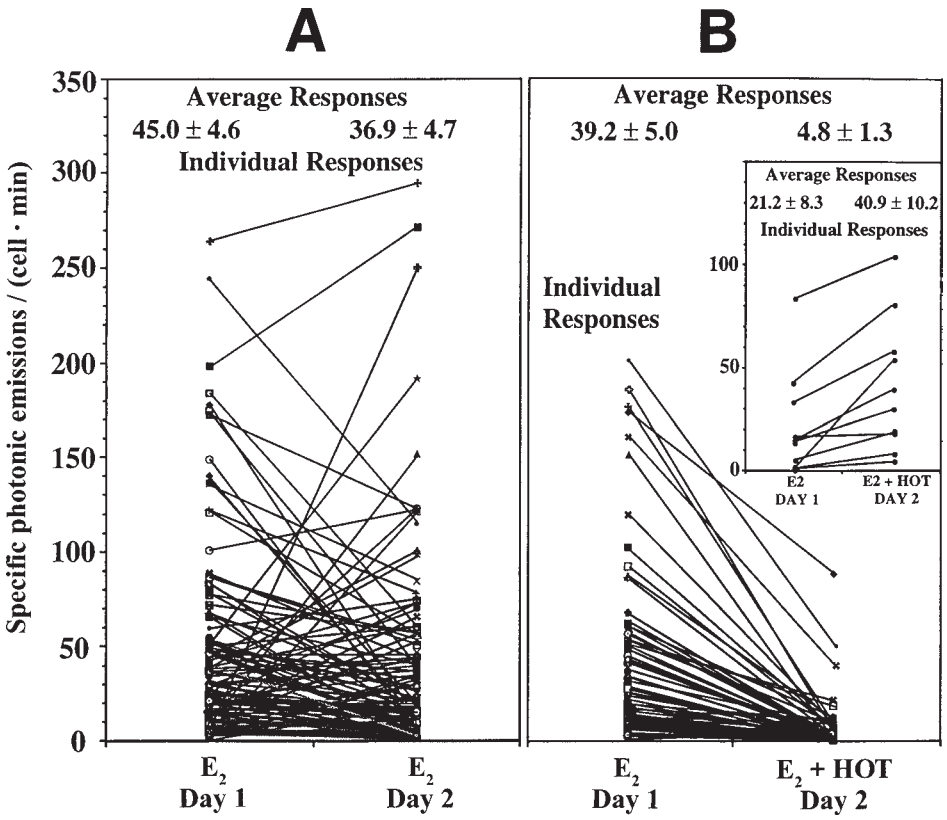

Fig. 3. Interactive effects of estrogen and antiestrogen on ERE-driven luciferase activity in T-47D cells. Cells transfected with the MMTV-ERE-LUC plasmid were subjected to photonic imaging after 24 h of treatment with E_2 (1 n*M*; d 1) followed by 24 h of exposure to E_2 or E_2 + HOT (1 μ*M*; d 2). (**A**) Cells (*n* = 121) treated continuously with E_2 exhibited elevated levels of photonic activity that fluctuated randomly from one day to the next. However, the average response did not change over time. (**B**) Cells (*n* = 93) exposed to E_2 for the initial 24 h (d 1) exhibited an increase in ERE-driven luciferase activity that was reversed in most cells (89.2%; main panel) following treatment with HOT. By contrast, HOT had no effect or actually stimulated ERE-driven reporter activity in other cells (10.8%; inset). This seemingly paradoxic effect of HOT on a minority subset of cells was masked when only the population (average) responses were considered.

emissions to cells treated on consecutive days with E_2 alone (Fig. 3A). Collectively, these data suggest that a minority subpopulation of T-47D cells that responded positively to the effects of E_2 were not inhibited by the addition of HOT. Inasmuch as these cells had not been exposed previously to TAM or HOT, these results indicate that a subpopulation of these breast cancer cells possesses an intrinsic (as opposed to acquired) resistance to HOT.

Discussion

We previously described a novel paradigm for dynamic monitoring of E_2-regulated gene expression in single, living breast cancer cells *(9)*. The capacity of this single-cell strategy to reveal heterogeneous responses that might be obscured at the population level suggested its potential for identifying cells that were differentially responsive to therapeutic agents. That potential was realized in the present study when the system was used to explore whether HOT exerted its seemingly paradoxic effects (agonistic and antagonistic) on all or just subsets of breast cancer cells.

Our initial efforts were aimed at identifying cells in T-47D cultures that were intrinsically responsive to the partial E_2 agonist actions of HOT. We measured E_2-regulated gene expression in the same E_2-starved cells both before and after exposure to HOT. The analytical power afforded by using each cell as its own control enabled us to identify a subset of cells that was intrinsically responsive to the agonistic effects of HOT. At present, we are cautious about drawing conclusions on the relative size of this subpopulation for two reasons. First, we could not detect basal photonic emissions from the vast majority of transfected cells maintained in E_2-depleted medium. Thus, some of the cells may have exhibited positive responses to HOT that were still below our equipment's threshold of sensitivity. Second, it might be argued that our selection of a reporter construct that contained five tandem EREs provided an overestimate of the relative abundance of positive responders. Indeed, increasing the number of tandem EREs has been shown to enhance the E_2-agonist activity of HOT *(10,11)*, and therefore, this could have increased our sensitivity of

detection. These quantitative considerations notwithstanding, our results support the conclusion that the partial agonist effects of HOT are manifested on some but not all T-47D cells.

An additional aim of our investigation was to determine whether HOT could override E_2 induction of gene expression uniformly. Whole-population studies of T-47D cells showed previously that E_2-stimulated expression of the MMTV-ERE-LUC plasmid in T-47D cells could be inhibited by the addition of HOT *(12)*. In the present study, with single cells, we found a similar antiestrogenic effect of HOT when just the collective (averaged) responses of all cells in T-47D cultures were considered. However, examination of individual responses of cells cotreated with E_2 + HOT revealed a subset (10.8% of E_2 responders on the previous day) that was in fact stimulated by subsequent exposure to HOT. Such a finding is not unexpected in light of reports that extremely long-term treatment of breast cancer cells with TAM caused an apparent remodeling of the population to favor cells with a phenotype that is stimulated by TAM *(1,8)*. The significance of our observations in relation to this remodeling hypothesis is that we have identified individual cells that without previous exposure to HOT were intrinsically resistant to HOT's antiestrogenic effects. While any population of cells may exhibit varying degrees of heterogeneity in response to a hormone treatment (as seen with E_2) (Fig. 3A), it is that one cancer cell out of hundreds or even thousands that does not respond appropriately to antiestrogen treatment that represents the potential threat of tumor recurrence (Fig. 3B). Our data demonstrate the existence of a minority subset of cells with the potential for redefining a breast cancer population after the majority of cells that respond to antiestrogen in an appropriate manner have been eliminated therapeutically.

In summary, we have refined and utilized a strategy for assessing the responses of individual breast cancer cells to antiestrogen treatment. Our results reveal the existence of minority tumor cell subpopulations that are paradoxically stimulated by HOT when treated either alone or in combination with E_2. Whether these two subpopulations are one and the same or separate and distinct remains to be determined. Likewise, the issue of whether these responsive states are permanent or transitional for a given cell as well as the molecular basis for these phenotypic subtypes will require further investigation. Regardless of how these intriguing issues are resolved, our basic science observations pave the way for developing a clinical test for predicting responsiveness to endocrine therapies. To give just a single example, minor modifications to the present assay should enable us to establish, *a priori*, whether breast cancer cells from a given patient possess functional ERs, and if so whether TAM can attenuate the estrogen response. This strategy can be used to identify those breast cancer patients (one-third to one-half) who are ER positive and receive TAM but are not able to respond to the antiestrogen actions of the compound and therefore cannot benefit in terms of recurrence rates.

Materials and Methods

Culture and Transfection of T-47D Cells by Microinjection

Whole populations of ER^+ T-47D breast adenocarcinoma cells (American Type Culture Collection, Rockville, MD) were maintained as monolayers in phenol red–free RPMI-1640 medium supplemented with 0.2 IU of bovine insulin, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 10 m*M* HEPES, and 10% FBS (Life Technologies, Grand Island, NY) in a 95% air/5% CO_2 humidified environment at 37°C. Prior to an experiment, culture flasks containing the cells were washed with medium devoid of serum and subsequently exposed to RPMI-1640 supplemented with the same percentage of FBS that had been charcoal-stripped to remove estrogen (cs-FBS). Twenty-four hours later, flasks were trypsinized (0.05% trypsin/0.53 m*M* EDTA) (Life Technologies) to yield a suspension of cells for culture on cover slips photoengraved with a numbered and lettered grid pattern. These gridded cover slips were used to facilitate reidentification of individual T-47D cells on consecutive days of an experiment. Cells were plated onto the cover slips at a concentration of 30,000 cells in 300 μL of RPMI-1640 without FBS and incubated for 1 h to achieve attachment of the cells. Then, 1.7 mL of RPMI-1640 + 10% cs-FBS was added to each 35-mm well containing a cover slip. After a 24-h incubation, cells on a particular grid of a cover slip were transfected by microinjection with an MMTV-ERE-LUC reporter plasmid. This MMTV-ERE-LUC plasmid (provided by D. McDonnell, Duke University Medical School, Durham, NC) contains five copies of the 33-bp vitellogenin ERE fused to the firefly luciferase gene. It has been used previously by our laboratory *(9,13)* and others *(14)* as an estrogen-responsive reporter. The MMTV-ERE-LUC construct was microinjected into single cells with the aid of a semiautomated microinjection system (Eppendorf, Madison, WI) in accordance with parameters previously established by our laboratory for breast cancer cells *(9)*. After transfection, cells were washed and immersed in medium containing no treatment, E_2 (1 n*M*), or HOT (1 μ*M*) for 24 h prior to imaging on d 1 of an experiment. A dosage of 1 μ*M* HOT was chosen for use as an experimental treatment for three reasons. First, this same dose is reported to suppress growth of ER^+ breast cancer cells *(15–17)*. Second, long-term incubation of cells with μ*M* concentrations of HOT has induced antiestrogen resistance in breast cancer cell lines *(18)*. Third, we found previously that a 1 μ*M* dose can block E_2 activation of the MMTV-ERE-LUC reporter plasmid in entire cultures of lipofectamine-transfected T-47D cells *(12)*.

Quantitative Photon Counting

Photonic emissions from individual, transfected T-47D cells were quantified as described previously *(9)* and as depicted in Fig. 1. Briefly, a gridded cover slip bearing the transfected cells was assembled into a Sykes-Moore cham-

ber that was subsequently filled with medium (Dulbecco's modified Eagle's medium/F12) containing 0.1 m*M* luciferin (luciferase substrate; Sigma, St. Louis, MO). The assembled chamber was then transferred to the heated stage of a photon capture system (Hamamatsu VIM Photon Counting Camera and Argus-50 Image Processor; Hamamatsu Photonics Systems, Bridgewater, NJ). After a bright-field image (for reference purposes) was captured, the lights were turned off and photonic emissions from individual cells were accumulated in 10-min bins and quantified (Fig. 1). On completion of the initial measurement (d 1), the Sykes-Moore chamber was disassembled, and the cover slip bearing the cells was washed and placed for an additional 24 h in the next appropriate treatment as follows: medium alone, E_2 (1 n*M*), HOT (1 μ*M*), or E_2 + HOT. The same cells imaged on d 1 were then relocated by their position on the gridded cover slip and subjected again to photonic imaging on d 2 (Fig. 1).

Statistical Analyses

Comparisons of means among treatment groups were made using the student's t-test, and data are expressed throughout as the number of specific photonic emissions per minute. The level of significance among treatment comparisons was established at $p < 0.05$.

Acknowledgments

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