Chapter 8

Rapid, High-Throughput Detection of Endocrine Disrupting Chemicals Using Autobioluminescent Cellular Bioreporters

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Abstract

Overexposure to endocrine disruptor chemicals (EDCs) can result in serious health problems, yet they are commonly found in everyday items such as pesticides, personal care products, nutritional supplements, and plastics. The U.S. Environmental Protection Agency, along with other such agencies from around the world, have therefore mandated that new approaches be designed to screen these products for the presence of EDCs. However, despite the presence of several types of extant EDC detection assays, there still exists a backlog approaching 87,000 chemicals currently awaiting screening. Autobioluminescent detection systems, which utilize cellular bioreporters capable of autonomously modulating bioluminescent signals without the need for external stimulation or investigator interaction, provide an attractive means for addressing this backlog because of their reduced performance costs and increased throughput relative to alternative assay systems. This chapter reviews the variety of existing EDC detection assays and evaluates the performance of a representative autobioluminescent estrogen-responsive EDC bioreporter to provide an overview of how autobioluminescence can be used to improve EDC detection using *in vitro* assay systems.

Keywords: bioreporter, autobioluminescence, high-throughput analysis, endocrine disruptor, estrogen, luciferase

1. Introduction

The human endocrine system is an interconnected, finely tuned network of glands that produce hormones responsible for health and well-being from the time of conception until death. Chemicals classified as endocrine disruptors (EDCs) interfere with the production, release, transport, and/or action of these hormones and cause imbalances that are suggested to result in significant negative health impacts such as infertility, premature puberty, obesity, diabetes, heart disease, and breast, prostate, testicular, thyroid, endometrial, and ovarian cancers [1]. These chemicals, which are present in a variety of sources including pesticides, cosmetics, and plasticizers, number in the tens of thousands (**Figure 1**) [2].

The potential adverse effects of EDCs on human, wildlife, and ecosystem health have received significant worldwide attention from the scientific community, regulatory agencies, and the general public. Unfortunately, the uncertainties inherent to understanding the true health consequences of EDC exposure have fostered significant controversy, and the lay person is besieged with an extensive collection of 'facts' when attempting to grasp the fundamental content of the EDC problem. One only needs to Google bisphenol-A (BPA) to appreciate the informational complexity surrounding a chemical suspected of being an endocrine disruptor. Capitalizing on the difficulties posed by this situation, a multitude of companies have formed to evaluate how the compounds that make up everyday items such as pesticides, personal care products, nutritional supplements, and plastics can imbalance the delicate regulation of normal endocrine function in humans and wildlife.

There are currently over 500 contract testing service companies in the U.S. alone that are dedicated to performing assays for the chemical, pesticide, and personal care products industries, and this industry is expected to continue growing year-over-year at an annual rate of 13.5% [3].

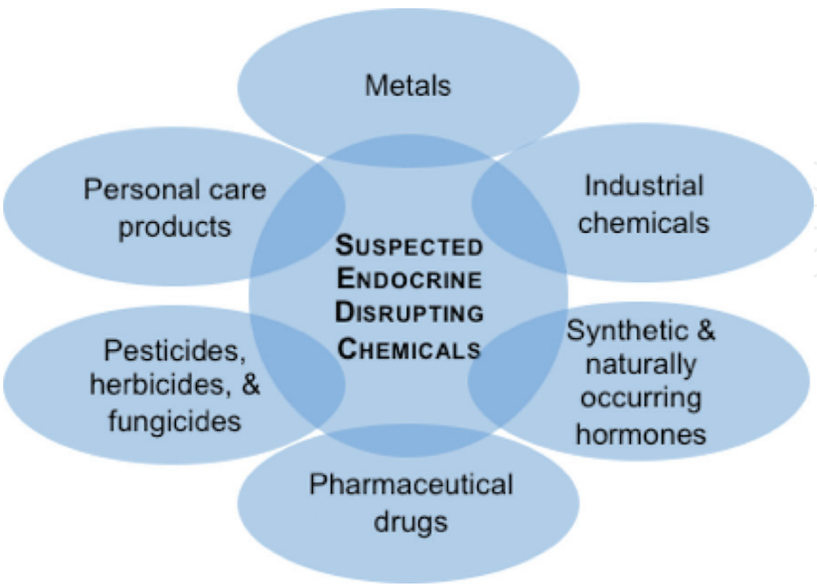

Figure 1. Tens of thousands of chemicals are suspected of having the potential to interfere with the endocrine system, resulting in adverse health effects in people and wildlife.

To improve throughput and decrease costs, these companies have adapted a two-tiered screening format, with Tier 1 consisting of *in vitro* assays aimed at identifying those chemicals that have the potential to interact with the endocrine system, and Tier 2 re-screening those compounds that test positive using *in vivo* assays to define their endocrine-related effects and obtain dosage-relevant information. Unfortunately, despite their societal importance, these tests remain biologically, logistically, and economically challenging. Tier 1 testing of chemicals for potential EDC activity is estimated to cost from $100,000 to $250,000 per chemical, with Tier 2 testing requiring upwards of 1,200 experimental animals and costing $1.2–$2.5 million per chemical [4, 5]. The majority of these costs will be borne by the chemical manufacturing industry, which then trickles down as increased prices at the consumer level. Furthermore, many of the common Tier 1 assay formats employed by these companies use non-human cell lines that can obscure bioavailability data [6, 7], require the use of radioactive materials that necessitate dedicated use areas and specially trained personnel [6–8], rely on expensive analytical equipment [8, 9], or do not meet the U.S. Environmental Protection Agency's (EPA) full testing requirements [3].

Realizing the deficiencies of these screening programs, and receiving considerable pressure from the public to reduce the use of animals for EDC testing, the U.S. EPA, with stakeholder input from the NIH National Institute of Environmental Health Sciences (NIEHS), has established the Endocrine Disruptor Screening Program for the twenty-first century (EDSP21) [10]. The goal of EDSP21 is to replace the current battery of Tier 1 tests with less expensive and faster high-throughput assays that can reduce the number of compounds that unnecessarily move forward to Tier 2 testing. This focus on improving the characterization of chemicals during Tier 1 screening is paramount to controlling costs, as mischaracterizations (i.e., false positives) during the Tier 1 stage magnify the costs of downstream Tier 2 screening, with a chemical's progression through multiple phases of Tier 2 screening only to be classified as negative for EDC activity representing a very poor return on investment. With the current chemical backlog approaching 87,000 chemicals [11], and considering the conventional scientifically acceptable false positive error rate of 5%, under current Tier 1 testing formats a minimum of 4350 chemicals will likely mistakenly proceed toward Tier 2 screening at a cost of approximately $8 billion.

The use of autobioluminescent EDC cellular bioreporters represents an attractive means to overcome the limitations of existing Tier 1 screening platforms and address the needs of the EDSP21 program. Autobioluminescence, with is defined as the ability to self-initiate the production of a luminescent signal using only endogenously supplied substrates to perform the enzymatic reactions necessary for signal generation [12], can reduce the number of required assay steps, eliminate the need for superfluous reagent costs, maintain human bioavailability relevance through the use of human cellular hosts, and increase throughput by minimizing hands-on performance time and employing automated processing and detection systems [13]. These benefits are made possible by the autonomous functionality of the synthetic luciferase gene cassette (*lux*) that controls the autobioluminescent phenotype. To enable autonomous EDC detection, *lux* cassette expression is regulated by a yeast upstream activating sequence (UAS), which is itself activated by a hybrid Gal4 transcriptional activator. Expression of this activator is, in turn, governed by the binding of an EDC to

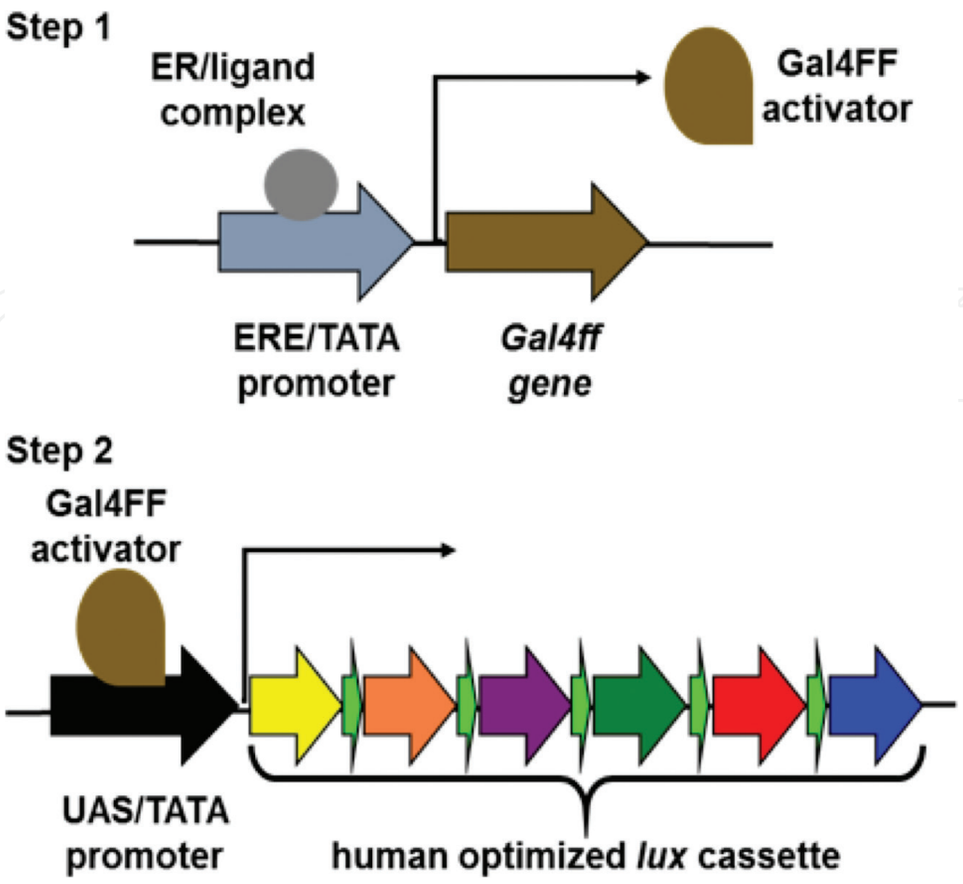

Figure 2. Functional schematic of an estrogenic compound-responsive autobioluminescent induction system. Step 1: *Gal4ff* expression is induced upon estrogenic compound exposure. Step 2: The *lux* cassette is then activated through stimulation of the UAS/TATA promoter by the *Gal4FF* transcriptional activator. Androgenic compound induction proceeds similarly.

an upstream estrogen (ERE; pictured) or androgen (ARE) response element (**Figure 2**). The use of this EDC-responsive promoter system within a human cell can therefore signal EDC bioavailability while simultaneously providing information regarding the timing, magnitude, and duration of the resulting effect. Using the detection of estrogenic compounds as an example, this chapter will provide an overview of how these autobioluminescent cellular bioreporters function in this role relative to alternative, traditional Tier 1 EDC sensor platforms and the advantages and disadvantages they provide for addressing the needs of the EDSP21 program.

2. Requisite endocrine disrupting chemical detection parameters

The U.S. EPA [14] and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [15] have established performance requirements for all EDC detection assays. These performance requirements ensure that the assays can function efficiently enough to identify the presence of EDCs at levels believed to be impactful to human and environmental health. However, rather than mandating that an assay demonstrates predetermined responses

Compound	Log PC_{50}	Log PC_{10}	Log EC_{50}	Hill Slope	Concentration
17β-estradiol	-11.4 ~ -10.1	< -11.0	-11.3 ~ -10.1	0.7 ~ 1.5	$10^{-14} \sim 10^{-8}$ M
Cortisol	-9.6 ~ -8.1	-10.7 ~ -9.3	-9.6 ~ -8.4	0.9 ~ 2.0	$10^{-12} \sim 10^{-6}$ M
17α-Methyltestosterone	-6.0 ~ -5.1	-8.0 ~ -6.2	N/A	N/A	$10^{-11} \sim 10^{-5}$ M
Corticosterone	N/A	N/A	N/A	N/A	$10^{-10} \sim 10^{-4}$ M

PC_{50}—concentration which induces a response at 50% of the maximal positive control response.
PC_{10}—concentration which induces a response at 10% of the maximal positive control response.
EC_{50}—half maximal effective concentration.
EC_{50}—half maximal effective concentration.

Table 1. EPA guidelines for the successful detection of endocrine disruptor chemicals in human cells.

Compound	Average EC_{50} Value	Required Detection Range
Levonorgestrel	0.984 nM	0.689 ~ 1.279 nM
Cortisol	0.043 μM	0.030 ~ 0.056 μM
Hydroxyflutamide	41.5 μM	29.0 ~ 54.0 μM
Flutamide	N/A	Undetected

Table 2. ICCVAM average EC_{50} value guidelines required for the successful detection of androgenic compounds.

across all known EDC compounds, these organizations require that the assay respond appropriately to treatment with serial dilutions of representative strong, weak, and very weak agonists, and that they do not respond to an appropriate negative control. To be considered successful, estrogenic detection assays must meet the U.S. EPA metrics presented in **Table 1** and androgenic detection assays must meet the ICCVAM metrics presented in **Table 2**.

3. Non-autobioluminescent detection assay formats

There are five *in vitro* assay formats, other than autobioluminescence, that are used for EDSP21 Tier 1 screening [16] (**Table 3**). All of these assays are well-established, having been initially developed as early as the 1960's. Although their performance is reproducible and reliable, each is subject to a number of detriments that limit their utility for low-cost, high-throughput EDC detection with high human relevance [6–9, 14].

For instance, in the estrogen receptor (ER) binding assay, cytosol must be isolated from the uteri of rats that have undergone ovariectomy prior to collection of the uterine tissue. These animal subjects are ovariectomized 7–10 days before harvesting the uterine tissue, with each test chemical requiring the use of approximately 19 subjects. Once the uteri have been harvested, they are homogenized and centrifuged to isolate ER-containing cytosol. Before conducting the assay, saturation radioligand binding assays using various concentrations of radioactively labeled 17β-estradiol added to each batch of cytosol are performed to first

Assay	Detection Format	Direct human bioavailability?	Multi-day?	Requires Animal Subjects?
Estrogen receptor (ER) binding assay	Radiological	No	Yes	Yes
Androgen receptor (AR) binding assay	Radiological	No	Yes	Yes
Aromatase assay	Radiological	Yes	No	No
Steroidogenesis assay	Metabolite quantification	Yes	Yes	No
Estrogen receptor transactivation assay (ETRA)	Luminescence	Yes	Yes	No

Table 3. The five traditional ***in vitro*** tier 1 EDC detection assays used in EDSP21.

validate that there are sufficient ER concentrations and to confirm that the receptor is functioning with appropriate affinity. Only after this series of preliminary steps are the actual assays run. During the assay, radioactively labeled 17β-estradiol, uterine cytosol, and test chemical are combined and must undergo a 16–20 h incubation at 4°C in the dark. Following incubation, hydroxyapatite is added, and multiple washings are performed before a final elution with ethanol and measurement of radioisotope activity in a liquid scintillation counter [7]. Similarly, the complementary androgen receptor (AR) binding assay follows the same intricate assay steps as the ER binding assay, but begins with the collection of rat ventral prostate tissues using subjects that are castrated ~24 h prior to assay initiation. Similar to the ER binding assay, this inclusion of approximately 19 animal subjects per test chemical results in increased moral, economical, and logistical concerns [6].

Like the ER and AR binding assays, the aromatase assay also uses radioactively labeled chemicals as detection targets. In addition, assay performance also requires the use of controlled substances, and therefore necessitates specialized waste disposal. Although these attributes do not directly hinder assay performance, they add cost and increase the logistical hurdles underlying assay execution. However, the tradeoff for the use of these chemicals is an increased throughput. Under standard conditions, the aromatase assay can be completed with only 6–8 person hours per run. This makes the aromatase assay a more attractive format for companies concerned with personnel costs. Another advantage of the aromatase assay is that it uses human recombinant microsomes as the detection vehicle, which provides additional human bioavailability relevance compared with the use of animal tissues in the ER and AR binding assays [8].

Unlike the above-mentioned assays, the steroidogenesis assay uses a human adrenocortical carcinoma cell line as its detection vehicle, which provides direct information on the human-relevant effects of compound exposure. However, while this represents a significant advantage, it also comes with the drawback that the cells must remain exposed to the test

chemical for 48 h, making this one of the longer duration assay formats. Further complicating the throughput of the assay is the detection method, which uses liquid chromatography positive atmospheric pressure photoionization tandem mass spectroscopy (LC/APPI-MS/MS) to measure the hormone concentrations in the medium as the assay endpoint. While this provides exquisite levels of sensitivity, the equipment required to perform these measurements is relatively expensive and requires highly-skilled technical personnel for operation. This limits the performance of this assay format to only those labs large enough to justify the associated operational costs [9].

The estrogen receptor transactivation assay (ERTA), also uses a human cell line as its detection vehicle. In this case, the assay leverages a human cervical cancer cell line containing a firefly luciferase reporter gene that emits a bioluminescent signal when chemicals bind to and activate the estrogen receptor. The bioluminescent output of this format makes it an attractive option because it does not require specialized equipment or skilled personnel to perform. The cells for this assay are simply plated in microtiter plates, the test chemical is added, and the plates are incubated for 20–24 h. Following incubation, the luciferase assay reagent is then added to each well to lyse the cells, and bioluminescence is measured. While this assay format is among the most simplistic to perform, the multi-day performance period and the need for requisite sample destruction concurrent with the addition of an exogenous activation chemical impart concerns relating to throughput, performance costs, and the potential interaction of the activating chemical with the compound under study. Nonetheless, the ETRA remains a popular choice for EDC detection due to its many advantages relative to the alternative assay formats [14].

4. Autobioluminescent detection assay formats

4.1. Advantages

Autobioluminescent assays systems address the backlogging problems endemic to EDSP21 because they utilize human cellular hosts as their detection vehicles, their signal generation is fully performed by these host cells without the need for external stimulation, their resulting reporter signal does not require cellular destruction or interfere with cellular metabolism, they are capable of self-regulating bioluminescent production throughout EDC exposure, and they maintain the same output format (luminescent production) as the commonly used ERTA [13]. Because these same output and detection vehicle formats are maintained, autobioluminescent assay systems share the advantages of providing direct human bioavailability information and not requiring specialized equipment or skilled personnel to perform. However, unlike the ERTA, the bioreporter cells used in autobioluminescent assays do not require lysis and therefore remain viable for an unlimited number of repeated or fully continuous measurements. This allows cytotoxicity measurements to be taken on control wells within each plate at any time point desired and eliminates the need for duplicate plate preparation. Since all data are obtained in real-time, the assay intervals employed in autobioluminescent assays can be shortened or lengthened on-the-fly based on the results being obtained, which provides an increased level of flexibility when working with previously uncharacterized compounds

(**Figure 3**). In addition, the detection equipment used to perform the ERTA can be used to perform autobioluminescent assays, so no change in equipment infrastructure is required [17].

This continuous imaging ability of autobioluminescence provides higher levels of data acquisition than the alternative assay formats and is more amenable to high-throughput use. This results in a significant cost savings of approximately 87% per assay relative to the ERTA, which has the lowest performance costs of the alternative assay types. For example, under moderate throughput conditions a 96-well microtiter test plate can be used to accommodate triplicate replicates of four test chemicals and their associated controls. Using this testing format, it would require 21,750 96-well plates to characterize the existing backlog of 87,000 chemicals that are pending under EDSP21 [11]. Based on existing market costs for technician time and chemical reagents [18, 19], it would cost approximately $1.5 billion (USD) to process all of these compounds. However, the reduced performance costs of the autobioluminescent assay format, which result primarily from a reduction in technician hands-on time and removal of the need to purchase an activating chemical substrate, reduces these costs to approximately $191,000 (USD), representing a savings of approximately $1.3 billion (USD).

4.2. Performance and EDC detection abilities

To evaluate the utility of autobioluminescence's repeated interrogation approach, autobioluminescent T47-D cells were seeded in triplicate into multi-well plates and incubated under standard growth conditions for 24 h. After this time, the medium was removed, cells were washed once with phosphate buffered saline (PBS), refreshed with EDC-free medium, and

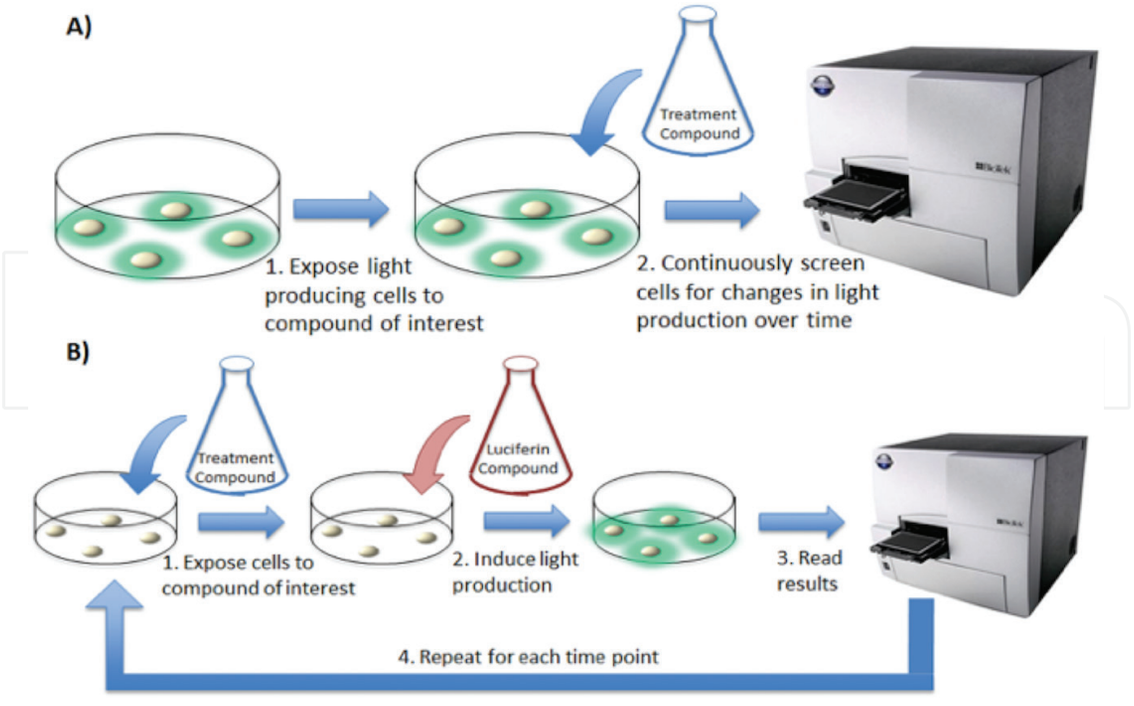

Figure 3. (A) The continuous signal generation of autobioluminescent assays allows for uninterrupted, real-time, high-throughput monitoring of cell activity across consecutive time points. This increases flexibility relative to (B) the ERTA, which only generates single time point snapshots of cellular activity.

supplemented with 17β-estradiol at concentrations of 0 pM (control), 0.1 pM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, or 100 nM. Autobioluminescent measurements were then obtained every 24 h for 6 days using an IVIS Lumina imaging system with a 10 min integration time. Increased autobioluminescent signals relative to untreated control cells were observed by day 3 for all treatments ≥1 pM, although this trend was only maintained throughout the full 6 day assay period at treatment levels ≥10 pM. A dose–response relationship was observed between 17β-estradiol treatment levels and autobioluminescence, with an EC_{50} value of 10 pM (**Figure 4**). Similar results were obtained using the alternative MCF-7 breast cancer cell line, which could detect 17β-estradiol at concentrations of both 1 and 10 nM through the significant ($p < 0.05$) induction of an autonomously-regulated autobioluminescent signal compared to both background light detection and the signal generated cells treated only with vehicle controls (**Figure 5A**).

Notably, the autobioluminescent production from both of these breast cancer cell lines displayed a relatively low signal-to-noise ratio, likely due to their natural expression of estrogen receptors and EDC transporters. To overcome this limitation, the system was re-created in the naturally ER-negative HEK293 human kidney cell line and co-transfected with human estrogen receptor alpha. This allowed for expression of the system without interference from native EDC uptake and processing pathways and significantly reduced the level of background autobioluminescent production in the absence of EDC stimulation, as well as

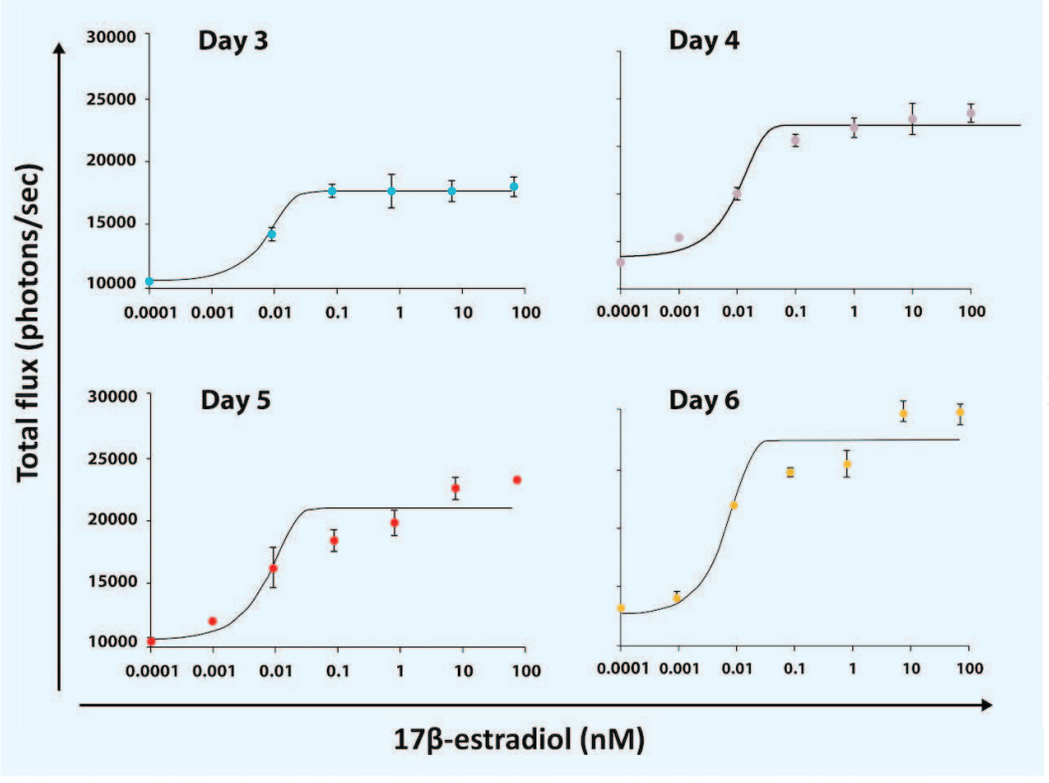

Figure 4. Using repeated measurements of T47-D breast cancer cell line samples, the autobioluminescent assay format allowed dose/response relationships between autobioluminescence and EDC treatment levels to be determined for each day that showed a significant increase compared to negative control cells.

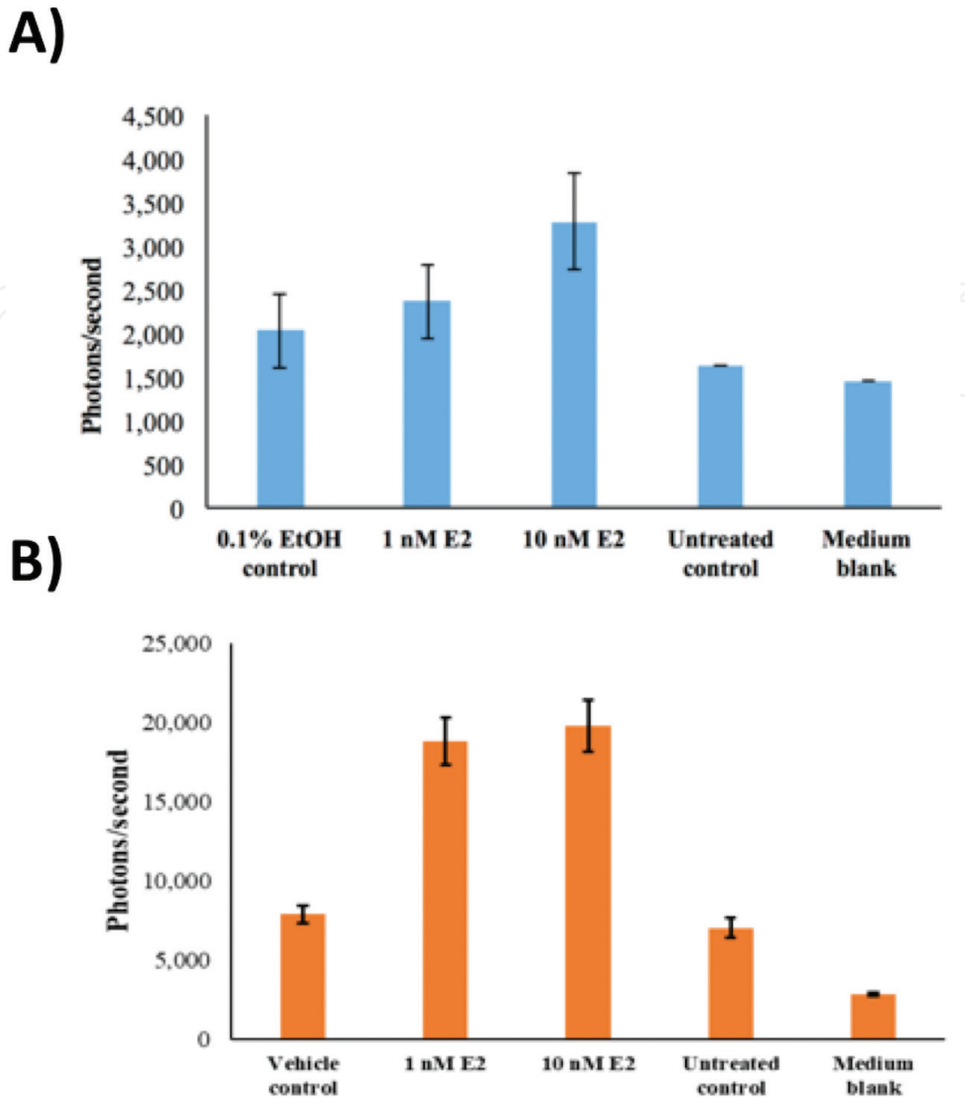

Figure 5. (A) An autobioluminescent MCF-7 cell line was capable of fully autonomous 17β-estradiol detection but displayed a low signal-to-noise ratio. (B) Re-created HEK293 cell line expressing human estrogen receptor alpha and the autobioluminescent reporter construct allowed for fully autonomous 17β-estradiol detection with an improved signal-to-noise ratio.

increasing the signal-to-noise ratio during positive detection events (**Figure 5B**). Using this system design, EDC-responsive autobioluminescent HEK293 bioreporters were able to detect an array of representative EDCs at levels relevant to the requirements of EDSP21 (**Table 4**).

This bioreporter similarly proved to be effective for the detection of other commonly encountered EDCs, such as synthetic hormones, synthetic industrial compounds, phytoestrogens, and fungicides (**Table 5**). These detection capabilities are especially promising given that the autobioluminescent system can be scaled to allow for robotic integration. This would allow cell plating, dosing, and reading to be fully automated. Since the addition of exogenous substrate or sample manipulation post-treatment is not required, this system reduces assay complexity and facilitates rapid detection using automated systems. Given its advantages relative to the existing assay formats (**Table 6**), autobioluminescence represents an attractive alternative assay for potential high-throughput Tier 1 screening of the EPA's current chemical inventory list.

Compound	Measured EC_{50} Value
17β-estradiol	7.9 pM
17α-estradiol	290 pM
17α-methyltestosterone	1 μM
Corticosterone	Negative

Table 4. When expressed in HEK293 cells, the estrogen compound-responsive autobioluminescent reporter system detected an array of representative EDCs within the EPA detection guidelines.

Compound	Compound Class	Measured EC_{50} Value
Diethylstibestrol	Synthetic hormone	12 pM
Bisphenol A	Synthetic compound	460 nM
Daidzein	Phytoestrogen	470 nM
Fenarimol	Fungicide	10 μM

Table 5. The autobioluminescent HEK293-based estrogenic compound-responsive bioreporter was found to be an efficient and simplistic means for the detection of a wide variety of compounds with known estrogenic effects.

Method	Uses non-human cells?	Requires chemical substrate?	Requires radioactive substances?	Requires specialized personnel?	Requires analytical equipment?	Scalable for high-throughput screening?
ER/AER binding	Yes	No	Yes	Yes	No	No
ETRA	No	Yes	No	No	No	No
Aromatase	No	No	Yes	Yes	No	No
Steroidogenesis	No	No	No	Yes	Yes	No
Autobioluminescence	No	No	No	No	No	Yes

Table 6. Summary of the observed advantages and disadvantages of the autobioluminescent EDC detection format relative to alternative tier 1 screening methods.

5. Future directions and recommendations

While autobioluminescent assays have the potential to significantly improve the throughput and cost effectiveness of Tier 1 EDC detection, they are currently in their infancy. Of the tested methods, only the HEK293-based autobioluminescent assay format was capable of producing data with similar performance metrics to the incumbent screening procedures. It is clear that the utility of the autobioluminescent assay format will need to expand to additional cell types and to the detection of androgenic compounds in order to fully address the bioavailability and health effects of EDCs. Similarly, while this work screened the performance of

the HEK293-based estrogen-responsive bioreporter against a variety of EDCs and associated controls, it will be necessary to validate the performance of this assay format at the levels of scale required for commercial use. Therefore, the development of additional bioreporter cell types and their validation at scale using automated assay preparation, performance, and detection equipment is recommended as a next step in the maturation of this assay format. If autobioluminescent assays can perform reliably under these conditions while maintaining a similar level of performance to that observed from the HEK293-based estrogen-responsive bioreporter, they will prove a valuable tool for Tier 1 EDC detection.

6. Conclusions

Tier 1 *in vitro* assays are the front line in EDC detection. However, the limitations of traditional assay formats, which use non-human cell lines that can obscure bioavailability data [6, 7], require the use of radioactive materials that necessitate dedicated use areas and specially trained personnel [6–8], or rely on expensive analytical equipment [8, 9], are currently incapable of handling the sheer number of compounds that must be screened. Autobioluminescent assays, such as the HEK293-based estrogen-responsive bioreporter assay presented here, are uniquely positioned to overcome the limitations of existing assay formats by autonomously generating bioluminescence in response to target chemical or chemical class bioavailability. The use of these reporter systems allows bioluminescent responses to be linked to EDC detection for reagent-free, fully automated screening at a fraction of the cost of existing assays, providing a promising route toward addressing the existing EDC compound screening backlog.

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Conflict of interest

S.R., G.S., and D.C. are board members in the for-profit entity 490 BioTech.

Abbreviations

AR	Androgen receptor
ARE	Androgen response element
BPA	bisphenol-A

EC_{50}	Half maximal effective concentration
EDC	Endocrine disruptor chemical
EDSP21	Endocrine Disruptor Screening Program for the twenty-first century
EPA	U.S. Environmental Protection Agency
ER	Estrogen receptor
ERTA	Estrogen receptor transactivation assay
ERE	Estrogen response element
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
LC/APPI-MS/MS	Liquid chromatography positive atmospheric pressure photoion ization tandem mass spectroscopy
lux	Synthetic luciferase gene cassette
NIEHS	NIH National Institute of Environmental Health Sciences
PC_{10}	Concentration inducing a response at 10% of the maximal positive control response
PC_{50}	Concentration inducing a response at 50% of the maximal positive control response
PBS	Phosphate buffered saline
UAS	Upstream activating sequence

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References

[1] Schug T, Janesick A, Blumberg B, Heindel J. Endocrine disrupting chemicals and disease susceptibility. Journal of Steroid Biochemistry and Molecular Biology. 2011; **127**(3-5):204-215

[2] Bergman A, Heindel J, Jobling S, Kidd K, Zoeller R. State of the Science of Endocrine Disrupting Chemicals. Geneva, Switzerland: United Nations Environment Programme and the World Health Organization; 2012

[3] Morea S. Outside knowledge: The industry will thrive as research and development budgets rise. In: US IIROCROit, editor. IBISWorld Industry Report OD5708 Contract Research Organizations in the US; 2014

[4] Hecker M, Hollert H. Endocrine disruptor screening: Regulatory perspectives and needs. Environmental Sciences Europe. 2011;**23**(1):15

[5] Elder M. Early Toxicology: Markets and Approaches. New York, NY: Kalorama Information Market Intelligence Report; 2012

[6] Environmental Protection Agency. Androgen Receptor Binding (Rat Ventral Prostate Cytosol) Standard Evaluation Procedure. Washington, D.C.: Endocrine Disruptor Screening Program; 2011

[7] Environmental Protection Agency. Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC) Standard Evaluation Procedure. Washington, D.C.: Endocrine Disruptor Screening Program; 2011

[8] Environmental Protection Agency. Recombinant Microsomal Aromatase Assay Validation Study: Positive Control Study. Washington, D.C.: Endocrine Disruptor Screening Program; 2011

[9] Environmental Protection Agency. Steroidogenesis (Human Cell Line - H295R) OCSPP Guideline 890.1550 Standard Evaluation Procedure. Washington, D.C.: Endocrine Disruptor Screening Program; 2011

[10] Environmental Protection Agency. Endocrine Disruptor Screening Program for the 21st Century (EDSP21 work Plan)—The Incorporation of In Silico Models and In Vitro High Throughput Assays in the Endocrine Disruptor Screening Program (EDSP) for Prioritization and Screening. Washington D.C.: Office of Chemical Safety and Pollution Prevention, U.S. Environmental Protection Agency; 2011

[11] Vogel J. Tunnel vision: The regulation of endocrine disruptors. Policy Sciences. 2004; **37**(3):277-303

[12] Xu T, Conway M, Frank A, Brumbaugh A, Ripp S, Close D. Autobioluminescent cellular models for enhanced drug discovery. In: Chen T, Chai S, editors. Special Topics in Drug Discovery. Rijeka, Croatia: Intech Publishers; 2016. pp. 1-23

[13] Xu T, Ripp SA, Sayler GS, Close DM. Expression of a humanized viral 2A-mediated *lux* operon efficiently generates autonomous bioluminescence in human cells. PLoS One. 2014;**9**(5):e96347

[14] Environmental Protection Agency. Estrogen Receptor Transcriptional Activation (Human Cell Line—HeLa-9903) OCSPP Guideline 890.1300 Standard Evaluation Procedure. Washington, D.C.: Endocrine Disruptor Screening Program; 2011

[15] ICCVAM. ICCVAM Evaluation of *in vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. NIH Publication No. 03-4503. Research Triangle Park, NC: National Toxicology Program; 2003

[16] LeBaron M, Coady K, O'Connor J, Nabb D, Markell L, Snajdr S, et al. Key learnings from performance of the U.S. EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 *in vitro* assays. Birth Defects Research Part B-Developmental and Reproductive Toxicology. 2014;**101**(1):23-42

[17] Class B, Thorne N, Aguisanda F, Southall N, McKew JC, Zheng W. High-throughput viability assay using an autonomously bioluminescent cell line with a bacterial *lux* reporter. Journal of Laboratory Automation. 2015;**20**(2):164-174

[18] D(−)-Luciferin, ACROS Organics, Chemicals, Biochemicals, and Diagnostics [Internet]. Available from: https://www.fishersci.com/shop/products/d-luciferin-acros-organics-2/p-154155#?keyword=D-luciferin [Accessed: April 23, 2018]

[19] Mika A. 2017 Life Science Salary Survey [Internet]. Available from: https://www.the-scientist.com/?articles.view/articleNo/50701/title/2017-Life-Science-Salary-Survey/ [Accessed: April 23, 2018]

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