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Image-based high-content reporter assays: limitations and advantages

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Transcription factors are promising targets in many therapeutic areas, and reporter assays represent a mainstay of the cellular approaches utilized to study their functions. Traditional reporter assays lend themselves to screening applications, but do suffer from some disadvantages. During the past decade, the development of image-based high-content reporter assays has boosted transcription factor drug discovery and contributed to the understanding of their functions and molecular dynamics. This review summarizes and discusses the technical approaches currently employed in high-content reporter assays.

Introduction

Transcription factors (TFs) are DNA-binding proteins that recognize specific enhancer or promoter regions (cis-regulatory elements) and activate or downregulate the transcription of adjacent target genes [1]. TFs are key regulators of genetic programs and are often causally implicated in the pathogenesis of cancer and other diseases; therefore, they are attractive targets for drug discovery [2]. The great interest around TFs in basic research and drug discovery led to the development of a plethora of cell-based approaches to study their functions. Traditional reporter assays, based on the detection of exogenous proteins under the control of cis-regulatory elements of interest, are among the most pursued technologies available to study TFs [3]. In recent years, advances in microscopy automation, image analysis and fluorescent molecular tools have permitted the introduction of powerful complementary analytical approaches, such as high-content assays (HCAs) [4]. This review discusses the limitations and advantages of image-based high-content assays employed in TF studies and compound screening.

Transcription factors as drug discovery targets

It has been predicted that the human genome contains about 2500–3000 genes encoding transcription factors (http://dbd.mrc-lmb.cam.ac.uk/DBD/index.cgi?Home) that play fundamental roles in controlling cellular homeostasis as well as in coordinating genetic reprogramming in response to extracellular stimuli or during development. TFs encompass five superclasses and numerous families, classified on the basis of their sequence homology, but all contain at least two domains: a DNA-binding Domain (DBD), which interacts with DNA, and a Trans-activating Domain (TAD) that interacts with transcriptional co-regulators and forms complexes with RNA polymerases.

Transcription factors can be activated by extracellular stimuli (growth factors, hormones, cytokines, environmental changes) or genetic programs that eventually result in the coordinated expression of a set of target genes. Fig. 1 shows a schematic diagram with the main activating pathways and the molecular consequences of TF and Nuclear Hormone Receptor (NHR) activation.

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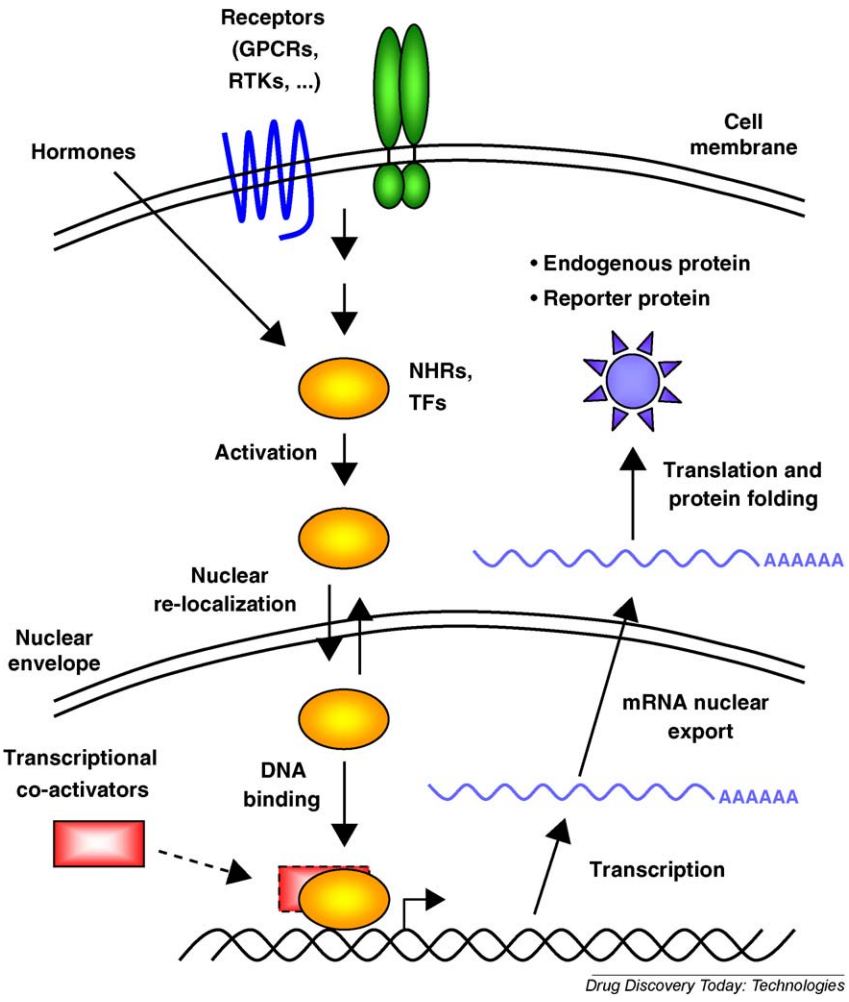

Figure 1. Transcription factor (TF) or nuclear hormone receptor (NHR) activation results in a series of molecular events leading to the modulation of expression of endogenous or artificial reporter proteins. Many of these events can be quantitatively monitored at the single-cell level by high-content assays. Membrane receptors, such as tyrosine kinase receptors (TRKs) or G protein-coupled receptors (GPCRs), can be activated by interaction with specific ligands (e.g. growth factors or cytokines) and in turn activate downstream signal transduction cascades, which eventually result in the activation (or inactivation) of cytoplasmatic transcription factors. Lipid-soluble steroid hormones, which diffuse through the plasma membrane, can directly bind to cytoplasmic nuclear hormone receptors. Activation of either cytoplasmatic transcription factors or nuclear hormone receptors occurs through diverse molecular events, such as phosphorylation, stabilization, dimerization, conformational changes or binding to other factors. A major consequence of these molecular events is the nuclear relocalization of transcription factors. It has been demonstrated that certain TFs, such as NF-κB, can undergo dynamic shuttling between the nucleus and cytoplasm. Once in the nucleus, activated transcription factors can directly bind DNA response elements and recruit transcriptional co-regulators to promote the transcription of endogenous or reporter genes. Messenger RNAs are exported to the cytoplasm and translated. Finally, protein expression requires correct protein folding: any drug that aspecifically impairs any of these late steps (e.g. protein synthesis or chaperonin inhibitors) might in principle interfere with reporter gene assays.

Deregulated, constitutively active TFs are often implicated in diseases, particularly cancer and other proliferative diseases. For example, it is known that the proliferation of a subclass of hormone-positive breast cancers is driven by Estrogen Receptor (ER) and Progesterone Receptor (PR), whereas Androgen Receptor (AR) is a main driver in primary prostate cancer [5]. Other transcription factors, such as the pro-apoptotic TFs belonging to FOXO family, are key downstream effectors of some the most deregulated signaling pathways in cancer (PI3K/Akt) [6]. Again, deregulated NF-κB signaling has been correlated to neoplastic transformation (reviewed in [7]). As a last example, p53 is a tightly regulated tumor suppressor, which is activated during cellular stress or DNA damage. p53 gene loss or inactivating mutations are found in more than 50% of human tumors [8,9]. Several pharmacological strategies are being developed for TF modulation: the most successful cases are certainly represented by the estrogen (e.g. Tamoxifen, http://www.astrazeneca.com/medicines/oncology/?itemId=3887970) and androgen (e.g. Casodex, http://www.astrazeneca.com/medicines/?itemId=3888008) NHR antagonists used in breast and prostate cancers, respectively. Various experimental strategies have been, or are currently being pursued to modulate activity of the

pharmacologically more challenging non-NHR TFs, including inhibition of activating kinases (e.g. Jak inhibitors for STAT family members, and IKK inhibitors for NF-κB) or modulation of TF stability (e.g. p53 protein stabilization through small molecule inhibition of p53-Mdm2 interaction [2]). Numerous biochemical and cellular techniques have been developed in the past decades to study TF function or to screen chemical libraries in search for TF modulators, but it is not the scope of this review to mention all. Rather, we will exclusively focus on cell-based reporter gene assays.

Reporter gene assays

Besides being extensively pursued to study gene regulation, promoter structure or signaling pathways, reporter gene assays (RGAs) represent one of the most commonly cell-based assays employed in drug discovery for compound and genetic screens.

Traditional RGAs are based on reporter genes, transiently or stably transfected into appropriate host cells in which the pathway of the TF of interest is functioning. Reporter genes encode for proteins whose expression is controlled by cis-regulatory elements, recognized by a given TF, and which can be easily assayed by means of their enzymatic activities or biophysical features as surrogate indicators of TF transcriptional activity. Among the most commonly used reporter genes are β-galactosidase, quantifiable through colorimetric readouts, cloramphenicol acyltransferase (CAT), monitored by liquid scintillation of CAT reaction products, and luciferase, which produces bioluminescence (reviewed in [3,10]). More recently, green fluorescent protein (GFP) has been employed as a reporter protein owing to a highly desirable characteristic: its quantification does not require cell lysis or substrate addition and allows non-invasive kinetic studies in living cells [11]. Regulatory elements upstream of the reporter gene might include known or putative promoters, or portions of them, or response and enhancer elements. RGAs generally assume that a DNA responsive element will be specifically recognized by a certain TF of interest. The *in vivo* situation is actually much more complex: it is known that many transcription factors competitively interact with the same response elements, affecting the interpretation of results for certain RGAs. For example, it is known that both ATF-2 and CREB bind cAMP/ATF Response Element regulating eNOS gene expression in endothelial cells [12]. Reporter protein stability and kinetics of induction/decay of reporter signal are additional important factors: whereas for end-point assays a stable reporter might be favorable, kinetic analyses ideally require reporter proteins with a rapid turnover rate. Clontech and others described, for example, the use of destabilized fluorescent proteins to decrease their half-life (http://www.clontech.com/products/detail.asp?product_id=10440&product_group_id=209289&product_family_id=1417&tabno=2) [11]. Among the factors influencing the detection of promoter activity in RGAs are the type of reporter protein, its basal transcription level, the expression vector used and the host cells [13]. Reporter gene assays are generally robust, sensitive, and have high dynamic ranges, but like all assays might suffer from artefactual or off-target interference; therefore, internal controls are highly desirable. For this purpose, companies providing reagents, vectors or cell lines for RGAs have developed assays that employ two distinct reporter genes under the control of an inducible promoter of interest and a constitutive promoter used as control for aspecific effects, respectively. Examples of dual reporter assays are the Dual-Luciferase® Assay (Promega, http://www.promega.com/catalog/catalogproducts.aspx?categoryname=productleaf_263) or the NovaBright™ β-galactosidase and firefly luciferase reporter assay (Invitrogen, http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Enzymes-and-Protein-Activity-Assays/EPAAssays-Misc/NovaBright-Chemiluminescent-Reporter-Gene-Assays/NovaBright-chemiluminescent-beta-galactosidase—luciferase-reporter-gene-assays.html).

Image-based high-content assays employed for TF analysis

High-content cellular assays usually employ automated microscopy or related technologies, coupled to image analysis and permit evaluation of multiple cellular responses to biological stimuli or drug treatment at the single-cell level. During the past decade, this technology has evolved to the extent that it is now well established and widely used in basic research and in drug discovery for compound and genetic screening (referred to as 'high-content screening', HCS) [4]. A major field of application of HCAs is the analysis of TF activity: some relevant examples from literature are summarized in Table 1. These examples span from single-cell kinetic studies of TF intracellular relocalization to high-content screening of large compound collections in search for selective TF inhibitors. Transcription factors that are objects of study by these HCAs encompass β-Catenin [14], members of the AP-1 complex [15–17], CREB [18], ERF1 [19], members of the FOXO family [6,20–24], HSF-1 [25], p53 [9,15], Stat3 [26], Androgen Receptor (AR) [5,27], Glucocorticoid Receptor (GR) [28], Vitamin D Receptor (VDR) [29] and NF-κB [30–35] (Table 1). Of note, many of these TFs are implicated in deregulated cell proliferation, but in any case they are all considered valuable pharmacological targets in oncology, immunomodulation or cardiovascular diseases. The majority of image-based high-content reporter screens described to date measure the nuclear translocation of few TFs belonging to the O subclass of forkhead transcription factors (FOXO) family [6,20–24]. Phosphorylation of FOXO family members by activated kinases of the PI3K/Akt pathway results in their inactivating retention in the cytoplasm, bound to regulatory molecules (i.e. 14-3-3 protein). PI3K pathway inhibitors, such

Table 1. Examples of high-content assays published in literature for the analysis of transcription factor regulation and activity

TF(s)[a]	TF family/function	Assay readout	Method of detection	Refs
β-Catenin	Transcriptional co-activator of Wnt pathway	Endogenous β-Catenin nuclear accumulation	IF[e]	[14]
SRF[b], Stats[c] and TCF[d]	Immediate early gene transcription factors	Endogenous c-Fos nuclear accumulation	IF[e]	[15,16]
c-Fos	Immediate early gene transcription factor (AP1 complex)	Endogenous c-Fos nuclear accumulation	IF[e]	[15,16]
c-jun	Immediate early gene transcription factor (AP1 complex)	Endogenous c-Jun phosphorylation (Ser63)	IF[e]	[17]
cAMP response element-binding (CREB)	Transcription factor (cAMP response element-binding)	CREB phosphorylation (Ser133)	IF[e]	[18]
Ets2 repressor factor (ERF1)	Transcriptional repressor of Ets family	Endogenous ERF1 nuclear accumulation GFP-ERF1 nuclear translocation	IF[e] RA[f]	[19] [19]
FOXO1 (FKHR)	Forkhead family transcription factor	FOXO1-FLAG nuclear translocation FOXO1-GFP nuclear translocation	IF[e] RA[f]	[6] [20,21]
FOXO3a (FKHRL1)	Forkhead family transcription factor	GFP-FOXO3a nuclear translocation	RA[f]	[22–24]
Heat shock factor protein 1 (HSF-1)	Heat-shock transcription factor	Endogenous HSF-1 nuclear stress granule formation	IF[e]	[25]
p53	TF activated during DNA damage or cellular stress response	Endogenous p53 nuclear accumulation EGFP-p53/Hdm2 GRIP[h] redistribution assay	IF[e] RA[f]	[15] [9]
Stat3	Stat family transcription factor	EGFP-Stat3 cytoplasm-to-nucleus translocation	RA[f]	[26]
Androgen Receptor (AR)	Nuclear hormone receptor	GFP-AR nuclear translocation AR-t1EosFP[i] nuclear translocation	RA[f] RA[f]	[5] [27]
Glucocorticoid Receptor (GR)	Nuclear hormone receptor	GFP-GR nuclear translocation	RA[f]	[28]
Vitamin D receptor (VDR)	Nuclear hormone receptor	VDR-GFP nuclear translocation	RA[f]	[29]
Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB)	TF activated during cellular stress response or cytokines	Endogenous p65 RelA nuclear translocation p65-dsRed nuclear translocation p50/p65 or p65/IκBα complexes formation in live cells	IF[e] RA[f] PCA[g]	[30] [31–34] [35]

[a] Transcription factor(s).
[b] Serum Response Factor.
[c] Signal transducer and activator of transcription.
[d] Ternary complex factor.
[e] Immunofluorescence.
[f] Redistribution assay.
[g] Protein-fragment complementation assays.
[h] GFP-assisted readout for interacting proteins.
[i] Fluorescent protein with UV-inducible green-to-red fluorescence conversion.

as Wortmannin, rapidly induce a decrease of FOXO phosphorylation levels, causing cytoplasm-to-nucleus relocalization. Given their role downstream in the pathway, and the fact that a macroscopic event such as a massive relocalization occurs when the PI3K/Akt pathway is inhibited, the intracellular localization of these TFs, *per se* or fused to fluorescent proteins, constitutes an optimal surrogate marker to report the status of activation of the whole pathway. A general property of cell-based assays, and of HCAs in particular, is the opportunity to investigate entire cellular pathways and subsequently characterize the specific targets of the hits identified (pathway dissection approach).

Another TF extensively studied by image-based high-content assays is NF-κB [30–35]. The activity of NF-κB complexes is negatively regulated by inhibitor proteins (i.e. IκBα): in non-stimulated cells, NF-κB proteins are sequestered to the cytoplasm bound to IκB proteins, which are themselves NF-κB target genes. Upon cellular stress or cytokine stimulation, IκB proteins are rapidly phosphorylated and degraded, allowing NF-κB activation and nuclear translocation. Subcellular localization of NF-κB complexes (in particular, of the subunit $p65^{RelA}$) has been exploited to screen for pharmacologically active compounds, as well as for basic NF-κB research. In this respect, it is important to mention that quantitative microscopy has played a fundamental role in delineating NF-κB dynamics in intact cells. In particular, intracellular localization time-lapse analysis demonstrated that NF-κB and IκBα undergo periodic oscillations of synthesis and nuclear translocation after cell stimulation. It has been showed that the frequency of these oscillations has a role in the definition of NF-κB target genes: as cytokine stimulation frequency is increased, late-gene transcription is differentially activated, indicating that the frequency of stimulation determines timing and specificity of expression of NF-κB target genes [31–34].

There are few examples in literature of comparative or parallel studies using image-based assays and traditional gene reporter assays. Among these, Nelson *et al.* describe a dual p65-dsRed and IκBα-EGFP expression/relocalization image-based analysis coupled to a NF-κB luciferase reporter assay in the same cells to study the role of activation and post-induction repression of NF-κB-dependent transcription [31]. A recent study by Unterreiner *et al.* reports the screening of a chemical collection with a high-content FOXO3a-GFP nuclear translocation assay in parallel with a luciferase reporter assay to measure endogenous FOXO3a transcriptional activity [23]. This group performed a comprehensive statistical analysis of the variability and sensitivity of these two assays. Surprisingly, they found only a small overlap of primary hits from the two assay types; in particular, HCA resulted more sensitive than luciferase reporter assay for the specific target and chemical collection employed. Proposed possible explanations for this discrepancy were as follows: (i) different compound incubation time between the two assays (1 h for HCA and 12 h for reporter assay); (ii) higher probability that non-specific compounds (false positives) could result active in the reporter assay with respect to HCA; (iii) possibility that unrelated transcription factors could activate the expression of luciferase reporter gene. Comparison of assay performances revealed higher robustness and sensitivity of the FOXO3a-GFP nuclear translocation assay with respect to the reporter gene assay.

Box 1. Pros and cons of using image-based high-content assays versus reporter gene assays

Pros

- Possibility to perform multiplexed, kinetic, single-cell analyses;
- Possibility to directly and quantitatively monitor TFs activation events in cells (phosphorylation, stabilization, protein–protein interactions, nuclear relocalization);
- Non-destructive approach (sample lysis is usually not required);
- The primary readout can be integrated with morphometric and cytotoxicity data;
- In some cases, HCA reported to show higher sensitivity and specificity with respect to the corresponding classical reporter assays [23];
- Unlikely classical reporter assays, readout signal magnitude is independent of the total number of cells in the sample;
- Possibility of an early identification of false positives in compound or genetic screens (e.g. toxic compounds).

Cons

- Low throughput of some HCS readers;
- In some cases, HCAs have lower signal-to-noise ratio and dynamic range with respect to corresponding RGAs [23];
- Laborious sample preparation, particularly for immunofluorescence assays;
- Cost of reagents (i.e. antibodies) or cell lines stably expressing FP constructs (a license agreement is usually needed for commercial cell lines);
- More complex data management and analysis.

The use of HCAs for TF analysis presents both advantages and disadvantages with respect to RGAs (detailed in Box 1).

Briefly, HCAs are non-destructive approaches that allow multiplexed analysis of TF function and localization in end-point or kinetic assays, therefore adding spatial-temporal dimensions to the simple readout of signal magnitude. A major drawback of HCAs is the fact that they are generally less sensitive and provide lower signal-to-noise ratios or dynamic ranges with respect to RGAs. Moreover, the relatively laborious sample preparation and the relatively low throughput of some HCS platforms has limited in the past their application for primary cell-based screening.

The most common biomolecular approaches described in HCA literature to analyze transcription factors are fluorescence-based and include (i) immunofluorescence (IF), (ii) redistribution assays (RA), (iii) protein-fragment complementation assays (PCA) and (iv) fluorescent protein (FP)-reporter

Table 2. Technologies employed for high-content reporter analysis: comparison summary table

	Detection of TF[a] expression levels, localization or post-translational modifications	Detection of TF complexes by reconstitution of functional FPs[b]	Detection of intracellular localization of TF-FPs fusion proteins	Detection of endogenous target protein expression	Detection of fluorescent reporter proteins
Technology type	Immunofluorescence	Protein-fragment complementation assays	Redistribution assays	Immunofluorescence	Fluorescent protein reporter assays
Selected technology companies	Abcam (http://www.abcam.com) Cell Signaling Technologies (http://www.cellsignal.com/ddt/hcs.html) Promega (http://www.promega.com) Santa Cruz Biotechnologies (http://www.scbt.com) Thermo Scientific - Cellomics (http://www.cellomics.com/) Invitrogen (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-and-Tissue-Analysis/Cellular-Imaging/High-Content-Screening.html)	Odyssey Thera (http://odysseythera.com/)	Thermo Scientific - BioImage (http://www.thermo.com/com/cda/article/general/1,21003,00.html) Panomics (http://www.panomics.com)	Abcam (http://www.abcam.com) Cell Signaling Technologies (http://www.cellsignal.com/ddt/hcs.html) Promega (http://www.promega.com) Santa Cruz Biotechnologies (http://www.scbt.com) Thermo Scientific - Cellomics (http://www.cellomics.com/) Invitrogen (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-and-Tissue-Analysis/Cellular-Imaging/High-Content-Screening.html)	Invitrogen (http://www.invitrogen.com/) Clontech (http://www.clontech.com/) System Biosciences (http://www.systembio.com) Panomics (http://www.panomics.com)
Pros	Possibility to virtually use any cellular model, given expression of TF of interest Allow analysis of expression levels and post-translational modifications (e.g. activating phosphorylation) of endogenous TFs	Low cost, lend themselves to automation Allow studies in living cells Allow direct visualization of protein–protein interactions (i.e. TFs or NHRs dimerization)	Low cost, lend themselves to automation Allow studies in living cells Particularly suited to multiplexing	Possibility to virtually use any cellular model Direct and physiological readout of TF target gene expression Do not require *a priori* knowledge of cis-regulatory and trans-regulatory elements of target genes	Low cost, lend themselves to automation Fluorescent reporter proteins allow kinetic studies in living cells and in vivo FPs do not require additional substrates or cofactors, unlike classical reporter assays Allow single-cell reporter assays Allow *in vivo* analysis of TF activity

Cons	The use of antibodies limits their application in primary cell screening Need of staining and multiple washing cycles	Low flexibility in terms of cellular models Potential clonal effects in cell lines stably transfected FP fragments fused to 'bait' and 'prey' proteins might interfere with their regulation, dynamics or interactions with other proteins	Low flexibility in terms of cellular models TF overexpression and fusion with FPs might interfere with its function or dynamics Potential clonal effects in cell lines stably transfected with the reporter plasmid Nuclear redistribution assays require nuclear envelope integrity and a functioning nuclear export machinery Sensitive to changes of cellular morphology	The use of antibodies limits their application in primary cell screening Need of staining and multiple washing cycles Readout might be affected by protein target stability or post-translational modifications	Low flexibility in terms of cellular models Possible clonal variation of FP expression in different stable clones Require *a priori* knowledge of cis-regulatory and trans-regulatory elements of target genes
Refs	[6,14–19,25–30]	[35,37,38]	[5,9,19–24,26–29,31–34]	[15,16]	[11]

[a] Transcription factor(s).
[b] Fluorescent protein(s).

gene assays. The relative merits of these approaches, together with a limited list of companies providing services or reagents for each, are reported in a comparison summary (Table 2).

Immunofluorescence

Immunofluorescence (IF) is widely employed in high-content analysis, because it can be applied to any cellular model expressing appropriate levels of the protein of interest. The main limitation of IF is the need for laborious and time consuming sample manipulation, including incubations with antibodies and multiple washes; moreover, it depends on the availability of validated antibodies and it is not commonly pursued for primary cell-based screening. Immunofluorescence has been often employed as a technique of detection in reporter high-content assays to monitor endogenous TF expression, localization or post-translational modifications, as well as to analyze the expression of TF target genes [6,14–19,25–30]. Here we report an example of IF-based high-content reporter assay from our own data. We starved NIH-3T3 cells by serum deprivation and followed the kinetics of c-Fos induction after serum stimulation (Fig. 2). C-Fos proteins are immediate early genes, and part of the AP-1 complex together with Jun proteins [36]. Because newly expressed c-Fos rapidly undergoes proteasomal degradation (protein turnover is fast), with our experimental setting c-Fos was considered as a reporter endogenous indicator of the activity of upstream TFs [i.e. Serum Response Factor (SRF), Signal Transducer and Activator of Transcription (STATs) and Ternary complex factor (TCF)], besides transcription factor itself. Data show a rapid increase in c-Fos expression starting from 15 min after serum stimulation, which reaches a maximum (>90% positive cells) between 1 and 4 h after serum addition. C-Fos IF signal decreases slowly at later time points, to return to basal levels at about 9 h after stimulation. The possibility to analyze single cells within a population, which is one of the most important advantages offered by high-content assays, in this particular experiment allows observation that both the kinetics and magnitude of c-Fos induction are heterogeneous among different cells of the same population. Importantly, IF does not require cell lines engineered with fluorescent constructs or artificial regulatory cis-elements or trans-elements, and this allows analysis of endogenous factors in a non-artificial setting.

Redistribution assays

High-content analysis or, in general, quantitative microscopy, nowadays represents the technique of choice to analyze the spatial-temporal kinetics of intracellular relocalization of transcription factor and the transcriptional effects on target genes. High-content assays that monitor complex cellular processes (e.g. activation of signal transduction pathways) using fluorescent protein intracellular relocalization as the primary readout (e.g. cytoplasm to

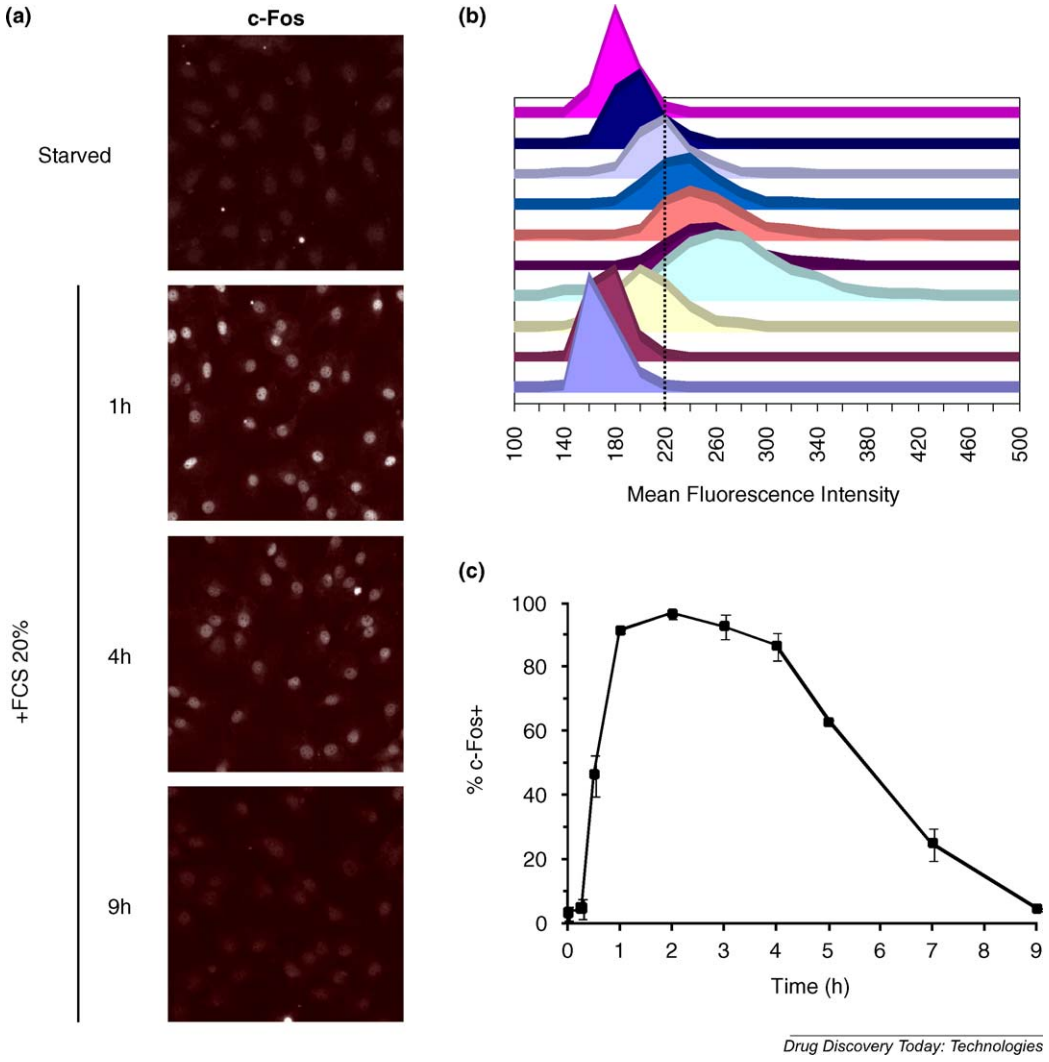

Figure 2. High-content analysis of endogenous c-Fos expression following serum stimulation in starved cells (authors' own unpublished data). NIH-3T3 cells seeded in 96-well plates were starved for 72 h by serum deprivation and stimulated at $t = 0$ with 20% v/v Fetal Calf Serum (FCS). Cells were fixed with formaldehyde 3.7% v/v at different time points after stimulation (from 15 min to 9 h), then immunostained with anti-c-Fos antibody (Santa Cruz Biotechnology) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). (a) Representative c-Fos immunofluorescence images of starved or serum-stimulated NIH-3T3 acquired by the ArrayScan V^{TI} HCS reader (Thermo Scientific Cellomics). Objective: 20×; exposure time: 0.5 s (DAPI images not shown). (b) C-Fos fluorescence intensity distributions at different time points, obtained by ArrayScan V^{TI} analysis. Dotted line represents a threshold arbitrarily set to define c-Fos positively stained cells (fluorescence intensity$>$ 220). (c) Percentage of cells positively stained for c-Fos at different time points, calculated from fluorescence intensity distributions shown in (b). The mean ±standard deviations of eight replicates are reported.

nucleus translocation of transcription factors-GFP fusion proteins) are commonly called redistribution assays (RAs). Many RAs have been developed to study the nuclear relocalization of TFs fused to fluorescent proteins such as NF-κB or FOXO proteins, as already mentioned, or ERF-1 [19], Stat3 [26] or NHRs [5,27–29]. An interesting redistribution assay variant is the GRIP technology (GFP-assisted readout for interacting proteins), which measures protein–protein binding (for example p53-Hdm2) taking advantage of the nuclear translocation analysis of non-bound prey protein to identify true interaction inhibitors (Thermo Scientific - BioImage) [9]. RAs are among the most used technique for compound and genetic high-content screening, with some successful cases: for example ETP-45658, the first pan-PI3K inhibitor entirely discovered and developed through HCS, was selected from a GFP-FOXO3a relocalization screening [24].

Protein-fragment complementation assays

Protein-fragment complementation assays (PCAs) are used to monitor protein–protein interactions in cells. As applied to high-content analysis, PCAs are based on fluorescence reconstitution by complementary fragments of fluorescent proteins (i.e. GFP) expressed as fusion proteins with two interacting proteins ('bait' and 'prey' proteins) (reviewed in [37]). This technique allows dynamic, direct visualization of protein–protein interactions in living cells, for example the

formation of NF-κB active and inactive complexes (p50/p65 and p65/IκBα) [35]. Several TF-interacting protein couples were reported, such as MAPK9:ATF2, Pin1:Jun, Elk1:Mapk1, ESR1:SRC-1, Mdm2:p53, ntCBP:p65, p53:Chk1, p53:p53, Pin1:p53 and Rad9:p53 [38].

Fluorescent protein (FPs)-reporter gene assays

To date, multipurpose plate readers are the instruments of choice for whole-well fluorescence measurements in GFP-reporter cellular assays. Nevertheless, HCS readers offer the possibility to analyze any fluorescent readout, including fluorescence expression of FP-reporter genes. Although infrequent, single-cell high-content approaches to analyze GFP induction in reporter cell lines have been described in literature. For example, a study by Li *et al.* described the simultaneous detection of NF-κB localization and NF-κB-mediated induction of a destabilized EGFP (dEGFP) created by fusing the C-terminal end of EGFP to the degradation domain of mouse ornithine decarboxylase (MODC) [11]. High-content analysis of FP reporters offers the advantage of combining single-cell, high multiplexing potential and direct visualization of a reporter gene (FP) without need for cumbersome sample manipulations. However, like any other RGAs, FP-reporter assays might not reflect the physiological regulation of the endogenous gene and generation of constructs requires *a priori* knowledge of cis-regulatory and trans-regulatory elements of target genes.

Conclusions

Image-based high-content assays currently pursued in industry and academia for transcription factor studies are principally oriented toward two scopes: (i) detailed investigation of the mechanisms of action of TFs, nowadays in synergy with systems biology approaches such as genome-wide gene suppression or chromatin immunoprecipitation (ChIP), to delineate how TF regulation and spatial-temporal dynamics influence target gene expression reprogramming; and (ii) identification of potential druggable mechanisms or targets in TF machineries, used as direct or surrogate markers to screening chemical or genetic (i.e. siRNA) collections. Both aspects will benefit from advances in HCA instrumentation, as well as in the 'molecular toolbox' of probes, fluorescent proteins and in cellular models (including primary cells, stem cells or isogenic cell pairs). In particular, ongoing efforts are aimed at improving both the sensitivity and robustness of image-based assays [4]. The introduction of novel molecular tools, fluorescent proteins or protein tagging technologies, such as HaloTag [39], will expand the possibilities of detection of protein modifications and protein–protein interactions in intact cell. Notably, the application of HCA to living cells has opened the way to sophisticated single-cell kinetic analyses of transcription factor activity and subcellular tracking of fluorescent reporters for a deeper comprehension of the cell-to-cell variability encountered in biological responses [34]. High-content assays in general, and in particular fluorescent engineered cellular models developed for TF analysis, lend themselves to be applied to whole organisms contributing to a 'translational imaging' approach. An example of this has appeared in a recent paper by De Lorenzi *et al.*, showing *in vivo* studies of NF-κB dynamics with GFP-p65 knockin mice [40].

On the drug discovery side, pharmaceutical research on TF inhibitors has already been successful in the past in delivering drugs with clinical activity, for example those acting as competitive nuclear hormone receptor antagonists. High-content screening is contributing to revitalizing the field of cell-based screening, already with some examples of compounds entirely discovered and developed by HCA [24], which show preclinical activity.

To date, the limited availability of comparative data between HCA and RGA assay performances renders premature any generalized consideration regarding the opportunity to preferentially use them in distinct contexts. Although some studies have reported discrepancies between the results obtained from these two techniques in defined screening contexts [23], it should nevertheless be borne in mind that such discrepancies might be expected from cell-based assays that, after all, investigate related but distinct biological processes (i.e. TF nuclear localization and consequent gene transcription). In the light of these considerations, both HCAs and RGAs can be considered viable and complementary approaches.

Related articles box

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