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.
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 of 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

![Diagram of 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 cytoplasmic transcription factors. Lipid-soluble steroid hormones, which diffuse through the plasma membrane, can directly bind to cytoplasmic nuclear hormone receptors. Activation of either cytoplasmic 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.]
pharmacologically more challenging non-NHR TFs, including inhibition of activating kinases (e.g. Jak inhibitors for STAT family members, and IKK inhibitors for NF-kB) 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 acetyltransferase (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 HCA 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 HCA encompass β-Catenin [14], members of the AP-1 complex [15–17], CREB [18], ERFI [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

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

$^a$ 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 p65ReLA) 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.

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 endpoint 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

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

<table>
<thead>
<tr>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possibility to perform multiplexed, kinetic, single-cell analyses;</td>
<td>Low throughput of some HCS readers;</td>
</tr>
<tr>
<td>Possibility to directly and quantitatively monitor TFs activation events in cells (phosphorylation, stabilization, protein–protein interactions, nuclear relocalization);</td>
<td>In some cases, HCAs have lower signal-to-noise ratio and dynamic range with respect to corresponding RGAs [23];</td>
</tr>
<tr>
<td>Non-destructive approach (sample lysis is usually not required);</td>
<td>Laborious sample preparation, particularly for immunofluorescence assays;</td>
</tr>
<tr>
<td>The primary readout can be integrated with morphometric and cytotoxicity data;</td>
<td>Cost of reagents (i.e. antibodies) or cell lines stably expressing FP constructs (a license agreement is usually needed for commercial cell lines);</td>
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<tr>
<td>In some cases, HCA reported to show higher sensitivity and specificity with respect to the corresponding classical reporter assays [23];</td>
<td>More complex data management and analysis.</td>
</tr>
</tbody>
</table>

In some cases, HCAs have lower signal-to-noise ratio and dynamic range with respect to 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. |
### Table 2. Technologies employed for high-content reporter analysis: comparison summary table

<table>
<thead>
<tr>
<th>Detection of TF expression levels, localization or post-translational modifications</th>
<th>Detection of TF complexes by reconstitution of functional FPs</th>
<th>Detection of intracellular localization of TF-FPs fusion proteins</th>
<th>Detection of endogenous target protein expression</th>
<th>Detection of fluorescent reporter proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technology type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunoﬂuorescence</td>
<td>Protein-fragment complementation assays</td>
<td>Redistribution assays</td>
<td>Immunofluorescence</td>
<td>Fluorescent protein reporter assays</td>
</tr>
<tr>
<td>Selected technology companies</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cell Signaling Technologies (<a href="http://www.cellsignal.com/ddt/hcs.html">http://www.cellsignal.com/ddt/hcs.html</a>)</td>
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<tr>
<td>Promega (<a href="http://www.promega.com">http://www.promega.com</a>)</td>
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<tr>
<td>Santa Cruz Biotechnologies (<a href="http://www.scbt.com">http://www.scbt.com</a>)</td>
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<tr>
<td>Thermo Scientiﬁc - Cellomics (<a href="http://www.cellomics.com/">http://www.cellomics.com/</a>)</td>
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<td></td>
</tr>
<tr>
<td>Pros</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Possibility to virtually use any cellular model, given expression of TF of interest</td>
<td>Low cost, lend themselves to automation</td>
<td>Low cost, lend themselves to automation</td>
<td>Possibility to virtually use any cellular model</td>
<td>Low cost, lend themselves to automation</td>
</tr>
<tr>
<td>Allow analysis of expression levels and post-translational modifications (e.g. activating phosphorylation) of endogenous TFs</td>
<td>Allow studies in living cells</td>
<td>Allow studies in living cells</td>
<td>Direct and physiological readout of TF target gene expression</td>
<td>Fluorescent reporter proteins allow kinetic studies in living cells and in vivo</td>
</tr>
<tr>
<td>Fluorescent reporter proteins allow kinetic studies in living cells and in vivo</td>
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</tr>
<tr>
<td>FPs do not require additional substrates or cofactors, unlike classical reporter assays</td>
<td>Allow single-cell reporter assays</td>
<td>Allow in vivo analysis of TF activity</td>
<td>Allow single-cell reporter assays</td>
<td>Allow in vivo analysis of TF activity</td>
</tr>
</tbody>
</table>

**Notes:**
- *a* Detection of TF expression levels, localization or post-translational modifications
- *b* Detection of TF complexes by reconstitution of functional FPs

**Sources:**
- 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/)
- Odyssey Thera (http://odysseythera.com/)
- Thermo Scientiﬁc - BioImage (http://www.thermo.com/cda/article/general/1,21003,00.html)
- Panomics (http://www.panomics.com)
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).
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/IkBα) [35]. Several TF-interacting protein couples were reported, such as MAPK9:ATF2, Pin1:Jun, Elk1:Mapk1, ESR1:SRC-1, Mdm2:p53, ntcpBP: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.

**References**