Development of a steady-state FRET-based assay to identify inhibitors of the Keap1-Nrf2 protein–protein interaction

Marjolein Schaap, Rowena Hancock, Andrew Wilderspin, and Geoff Wells*

University College London, UCL School of Pharmacy, London, United Kingdom

Abstract: One of the strategies proposed for the chemoprevention of degenerative diseases and cancer involves upregulation of antioxidant and free radical detoxification gene products by increasing the intracellular concentration of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). This can be achieved by disrupting the interaction between Nrf2 and Kelch-like ECH associated protein 1 (Keap1), a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex. Here, we describe the development of a high-throughput fluorescence (or Förster) resonance energy transfer assay for the identification of inhibitors of the Keap1-Nrf2 protein–protein interaction (PPI). The basis of this assay is the binding of a YFP-conjugated Keap1 Kelch binding domain to a CFP-conjugated Nrf2-derived 16-mer peptide containing a highly conserved “ETGE” motif. The competition aspect of the assay was validated using unlabeled Nrf2-derived 7-mer and 16-mer peptides and has potential as a screening tool for small molecule inhibitors of the PPI. We discuss the development of this assay in the context of other methods used to evaluate this PPI.

Keywords: FRET; Keap1; Nrf2; protein–protein interactions; high-throughput screening

Introduction
The development of therapies for the chemoprevention of cancer and chronic neurodegenerative conditions is an important, but challenging objective. In this respect, the regulation of cytoprotective responses in cells upon exposure to stressors is receiving growing interest as a therapeutic target. Fundamental regulators in this process include the substrate adaptor protein Kelch-like ECH associated protein 1 (Keap1) and the transcription factor nuclear factor erythroid related factor 2 (Nrf2). Nrf2 is able to upregulate the expression of numerous genes involved in protecting cells against...
carcinogenesis upon exposure to free radicals, electrophiles, or oxidative stress. Under basal conditions, Keap1 represses Nrf2 by targeting the transcription factor for ubiquitination and degradation by the proteasome. The Nrf2 Neh2 domain is responsible for binding to the Keap1 Kelch domain using both “low-affinity” DLG (residues 24–31) and “high-affinity” ETGE (78–82) motifs. These motifs interact with Keap1 mainly via salt bridges between Glu and Asp residues in Nrf2 and Arg residues in the Keap1 Kelch domain. Certain natural compounds (e.g., sulforaphane, curcumin) that activate Nrf2 are able to oxidize or covalently modify cysteine residues in the N-terminal broad complex, trammack, and bric-a-brac domain or intervening regions of Keap1. It is proposed that this causes a conformational change in the Keap1 dimer–Cul3 complex that inhibits ubiquitination of Nrf2.

An alternative strategy for upregulating Nrf2 involves disrupting the interaction between Keap1 and Nrf2 by direct competition at the protein–protein interface. Such an approach has potential advantages including a reversible inhibition mode and the possibility of increased target specificity. We and others have described the development of peptide inhibitors of the protein–protein interaction (PPI). More recently, small molecule inhibitors of the Keap1-Nrf2 interaction have been identified, although currently these are significantly less potent than the peptide inhibitors.

In this study, we describe the development of a homogeneous and high-throughput assay based on fluorescence or Förster resonance energy transfer (FRET) observed upon interaction between the Keap1 Kelch domain and a 16-mer Nrf2-derived peptide containing a high-affinity ETGE motif. The FRET technique is widely used and has the advantage of conjugating stable fluorophores to interacting proteins, which makes the assay relatively durable. The additional benefits of sensitivity and consistency makes this steady-state FRET assay well suited for high-throughput screening (HTS). The optimized FRET assay has been applied to quantify the binding activity of a range of Nrf2-derived peptides based on the high-affinity ETGE motif that serve as lead compounds for the development of potential inhibitors of the PPI. We discuss the relative merits of this assay in relation to fluorescence polarization (FP), surface plasmon resonance (SPR), and isothermal titration calorimetry (ITC) methods that have been applied previously.

Results

FRET optimization and validation

The principle of FRET is dependent on the overlap of the donor fluorescence emission spectrum with the acceptor excitation spectrum. In this case, the donor fluorophore is CFP conjugated to an ETGE motif-containing 16-mer Nrf2-derived peptide (CFP-Nrf2) and the acceptor is YFP conjugated to the human Keap1 Kelch domain (YFP-Kelch) [Fig. 1(a)]. When the two fusion proteins associate, the fluorophores are brought into close proximity (<10 nm). The crystal structure of the human Keap1 Kelch domain and interacting 16-mer Nrf2 peptide suggested that a fluorophore situated at either the C- or N-terminus of the Nrf2 peptide would be in close proximity. However, the FRET technique is widely used and has the advantage of conjugating stable fluorophores to interacting proteins, which makes the assay relatively durable. The additional benefits of sensitivity and consistency makes this steady-state FRET assay well suited for high-throughput screening (HTS). The optimized FRET assay has been applied to quantify the binding activity of a range of Nrf2-derived peptides based on the high-affinity ETGE motif that serve as lead compounds for the development of potential inhibitors of the PPI. We discuss the relative merits of this assay in relation to fluorescence polarization (FP), surface plasmon resonance (SPR), and isothermal titration calorimetry (ITC) methods that have been applied previously.
relatively close proximity (within 6–8 nm) to either the C- or N-terminus of the Kelch domain when the two proteins interact. The N-terminus of both proteins was selected for fluorophore conjugation to minimize potential steric hindrance when the two proteins associate. When the donor fluorophores are excited at 435 nm, a proportion of the absorbed energy is directly transferred to the acceptor. This results in a decreased emission of the donor at 475 nm as well as an increase in acceptor emission at 527 nm [Fig. 1(b)].

FRET resulting from the interaction of Nrf2 with Keap1 was demonstrated by a titration experiment in which emission spectra were recorded after the addition of YFP-Kelch at a range of concentrations to a fixed concentration of CFP-Nrf2 [Fig. 2(b)]. In a separate experiment, CFP-Nrf2 was titrated with unconjugated YFP to account for nonspecific interactions [Fig. 2(a)]. Also, emission spectra of the acceptor and donor fusion proteins were recorded separately to control for direct excitation of the fluorophores. As both fusion protein constructs have a TEV recognition site between the fluorescent tag and the protein, the FRET signal was further validated by the addition of ProTEV protease to a solution containing CFP-Nrf2 and YFP-Kelch, and as expected, this resulted in a rapid decrease in FRET (data not shown). The efficiency of FRET was quantified by measuring the decrease in donor emission at 475 nm for an optimum FE of 0.23. The FRET signal measured immediately after mixing the reagents and over a 24-h period was found to be stable throughout (data not shown).

In order to investigate factors modulating the observed FE, the effect of salt concentration in the buffer system was examined. X-ray crystallography studies of the complex formed between the Keap1 Kelch domain and the ETGE-containing Nrf2 peptide show that the interaction has a significant electrostatic component. This implies that high salt concentrations in the buffer may screen charge-charge interactions between Glu/Asp residues in the Nrf2 peptide and Arg residues in the Kelch binding site. Indeed, increasing the salt concentration (up to an additional 150 mM NaCl) in 20 mM Tris-HCl pH 7.4 buffer had a detrimental effect on the FE (100% FE at 0 mM NaCl decreased to ~80% at 50 mM NaCl, ~40% at 100 mM NaCl, and ~0% at 150 mM NaCl). Since DMSO is often used as a co-solvent for small molecules and peptides, a range of DMSO concentrations were tested in the assay system. Concentrations of 1% v/v were found to have a negative impact on the fluorophore emission spectra (data not shown). This limited the use of DMSO to a concentration of 0.1% v/v.

**FRET assay in a multiwell plate format**

Following optimization of the FRET system using the fluorescence spectrometer, the assay was adapted to a multiwell plate format. In this layout, an optimum FE was determined by titrating a fixed concentration of CFP-Nrf2 (CFP-WT) with variable concentrations of YFP-Kelch. A protein ratio of 0.11 μM CFP-Nrf2 and 0.20 μM YFP-Kelch was found to achieve ~80% of the maximal FE and was used subsequently in competition assays (Fig. 3). In order to demonstrate the specificity of the PPI, biologically relevant Nrf2-derived peptides that were known to exhibit a low binding affinity for the Keap1 Kelch domain were tested. These peptides incorporated mutations in the DLG (residues 24–31) and ETGE (residues 78–82) binding motifs that have been observed in certain human cancers. ITC studies suggested that mutations in the ETGE motif of Nrf2 such as E79Q, T80K, and E82D compromise the association with the Keap1 Kelch domain in...
cancerous tissue and cell lines, resulting in constitutive Nrf2 activation. CFP-peptide conjugates containing these three Nrf2 mutations were expressed and purified (CFP-E79Q, CFP-T80K, and CFP-E82D). FRET experiments were performed in which a fixed concentration of the CFP recombinant proteins was directly titrated with YFP-Kelch at a range of concentrations. All three mutant proteins showed a diminished affinity for the Kelch domain compared to the wild-type CFP-peptide conjugate (CFP-WT). In contrast, the affinity of the interaction can be improved by using a CFP-peptide conjugate with a double mutation E78P/F83L (CFP-E78P/F83L) that previously we have shown to increase binding affinity. Titration experiments with this construct show an improved interaction compared to the wild-type CFP-peptide conjugate (CFP-WT). In contrast, the affinity of the interaction can be improved by using a CFP-peptide conjugate with a double mutation E78P/F83L (CFP-E78P/F83L) that previously we have shown to increase binding affinity.

Next, we applied the assay to identify and characterize compounds that competitively inhibit the Keap1–Nrf2 interaction. As FRET is a sensitive measurement of the PPI, FE reduces when an inhibitor is added to the system. To verify this reduction in FE, fluorescence emission spectra were recorded in the absence and presence of 10 μM of an (unlabeled) Nrf2-derived peptide inhibitor (Fig. 4). The dose-dependence of the change in FE was used to identify Nrf2-derived peptide inhibitor (Fig. 4). The ability of this competition assay for HTS (see Supporting Information).

Unlabeled versions of the Nrf2-derived 16mer peptide sequences in Table I were used to further validate the suitability of the FRET assay for quantifying competitive inhibition of the Keap1–Nrf2 interaction. The results indicate that, as observed in the ITC experiments and our FRET studies using CFP conjugates, the three negative control peptides (E79Q, T80K, and E82D) were unable to perturb the association between CFP-Nrf2 and YFP-Kelch (Fig. 5). Moreover, the Nrf2 wild-type (Nrf2-WT) and E78P/F83L peptides caused a dose-dependent reduction in FE. The E78P/F83L peptide was a more potent inhibitor of the PPI than Nrf2-WT (IC50: 0.08 μM vs. 0.11 μM), consistent with the observations in Table I.

The standardized competition assay conditions were used to screen a series of previously described Nrf2-derived 7-mer peptides (Fig. 6). The decrease in peptide length (7mer vs. 16mer) had a significant effect on the binding affinity for Keap1. Truncation of the 16mer Nrf2-WT peptide resulted in peptide 1 with a ~30-fold higher IC50 value (3.34 μM vs. 0.11 μM). However, activity was improved after substitution of the N-terminal acetyl for a stearyl group in peptide 2 (0.45 μM vs. 3.34 μM). This favorable effect on affinity warrants further exploration. Peptides 3, 4, and 5 are proline-substituted versions of peptide 1 and show increased binding activity. This confirms our previous observations that the glutamate residue that precedes the ETGE motif is not essential for binding and that this position benefits from conformational restriction. Finally, peptide 6 is a scrambled version of peptide 2 and showed decreased binding by ~32-fold, which demonstrates the importance of an intact ETGE motif for interaction with the Keap1 Kelch domain in this assay. Overall, there is a close match between the IC50 values from the FRET assay and our previously described FP assay (Table II).

**Discussion**

Direct modulation of the Keap1–Nrf2 interaction is an emerging strategy to stabilize Nrf2 and induce cytoprotective gene expression. We have developed a high throughput steady-state homogeneous FRET assay that complements other assays applied to this PPI. FRET was observed by conjugating a CFP fluorophore to a 16-mer Nrf2-derived peptide and a YFP fluorophore to the Keap1 Kelch domain, which forms...
a specific binding signal that was validated by comparison with the unconjugated YFP control fluorescence. The CFP and YFP fluorescent proteins form a FRET pair with good spectral properties; additionally, conjugation to the proteins of interest simplifies the purification process of the fusion proteins, improves protein stability and increases the robustness of the assay. The ability to determine the absolute concentration of fluorophore conjugated proteins using their UV absorption and molar extinction coefficient improves the accuracy of dissociation constant measurements. For energy transfer to be possible, there needs to be a degree of spectral overlap between the donor and acceptor fluorophores. However, this spectral overlap can cause issues known as crosstalk or bleed-through. Excitation crosstalk occurs when the acceptor is excited with light at the excitation wavelength of the donor. Similarly, emission crosstalk arises from leakage of donor emission into the detection channel for acceptor emission. Determining FRET by quantifying the increase in YFP emission introduces the risk of including some emission bleed-through. We chose to use the decrease in donor emission as a direct measure of FE, as there is a reduced risk of spectral bleed-through in the CFP fluorescence emission channel.

The FRET method is distinct from a number of other techniques that have been applied to this interaction. It has a higher sensitivity and throughput than standard isothermal calorimetry and does not require the sample immobilization and significant method development that is needed for SPR. FRET has similarities to FP methods that have been applied successfully to this PPI by several groups in that the assay is homogeneous and high throughput. However, the FRET assay can be applied using less sophisticated plate readers with
Simpler optics.\textsuperscript{31,32} Both methods can be confounded by fluorescence interference from inhibitors, so secondary confirmation of hits is required in the context of HTS. A particular benefit of the FRET methodology in this case is the improvement in the stability of the Keap1 Kelch domain that results from conjugation to YFP. The time stability of this assay over FP approaches may be advantageous for HTS.\textsuperscript{16}

Some cell-based methods for identifying Nrf2 activating agents can be carried out in a relatively high-throughput manner (e.g., luciferase reporter\textsuperscript{33} and enzyme induction assays).\textsuperscript{34} However, they do not provide direct information on the mechanism by which Nrf2 transcriptional activity is induced. Recently, FRET techniques have been applied to the Keap1–Nrf2 interaction in single live cells using a multiphoton fluorescence lifetime imaging microscopy technique.\textsuperscript{35} This variation to the FRET methodology offers valuable insights into the protein interaction mechanisms in a cellular context; however, it is not yet amenable to a compound screening approach.

In summary, the FRET assay described here is a sensitive way to quantify protein–protein binding, with good signal stability and durability. Competition of (unlabeled) Nrf2-derived peptide inhibitors was successfully studied by determining the restored CFP fluorescence emission. These results provide an insight into peptide structure–activity relationships and complement previous FP studies by our group. Our ongoing work is focused on the application of this assay to the identification of small molecule inhibitors of the Nrf2 interaction. The developed FRET assay proves to be a valuable method for studying the Keap1–Nrf2 interaction and can aid in the identification and design of effective chemopreventive agents.

Materials and Methods

Materials

Primers were purchased from Eurofins, ProTev protease was supplied by Promega, and the 16-mer Nrf2-derived peptides were obtained from Peptide Synthetics.

Methods

**cDNA cloning, protein expression, and purification.** The cDNA encoding the human Keap1 Kelch domain (residues 321 – 609) was amplified by PCR with the following primers: 5'-AAAAAGATCCCGGC GCCAGTGGGCGG-3' (forward) and 5'AAAAGCG GCCGCTTAGTGACAGCCACGCCCAC-3' (reverse). The amplified product was digested with BamH1 and Not1 (NEB) and ligated into a pET28c-eYFP-TEV plasmid.\textsuperscript{18} The Nrf2 and Nrf2 mutant sequences (E78P/F83L, E79Q, T80K, and E82D) were created by annealing a forward and reverse primer (details on primers in Supporting Information). The annealed products were digested with BamH1 and Not1 and ligated into a pET28c-eCFP-TEV plasmid\textsuperscript{18} to generate the CFP-Nrf2 expression plasmids. All plasmid constructs were expressed in Escherichia coli Rosetta 2 (DE3) (MerckMillipore). A 1-L bacterial culture grown at 37°C was induced with 1 mM IPTG and incubated for 16 h at 21°C when the cells reached the exponential growth phase (OD600 nm = 0.4–0.6). The His-tagged recombinant proteins were purified by immobilized metal affinity chromatography on a 5-mL His-Trap column (GE Healthcare Life Sciences) (purification details in Supporting Information). All YFP-Kelch and CFP-Nrf2 or CFP-Nrf2 mutant protein concentrations were determined by UV/visible spectroscopy, using wavelengths of 514 and 435 nm for eYFP and eCFP, respectively, and the extinction coefficients 83,400 M$^{-1}$cm$^{-1}$ (eYFP) and 28,750 M$^{-1}$cm$^{-1}$ (eCFP). In the text, the terms eCFP and eYFP are used synonymously with CFP and YFP for simplicity.

**Fluorescence spectra.** All fluorescence spectra were acquired using a single sample unit PerkinElmer LS 55 luminescence spectrometer (5-nm slit width, 1-nm interval, 1-s integration) and an excitation wavelength of 435 nm. Fluorescence emission spectra were recorded from 400 to 600 nm. Samples were measured in cuvettes (3.5 mL volume, 10-mm path length, Sarstedt).

**FRET titration.** Titration experiments were performed using either a PerkinElmer LS55 luminescence spectrometer or a Pherstar BMG Labtech microplate reader (excitation filter: 430 nm, dual emission filters: 480 and 530 nm). YFP-Kelch or unconjugated YFP and CFP-Nrf2 or CFP-Nrf2 mutant proteins were diluted as appropriate in 20

---

**Table II. IC$_{50}$ Values for Nrf2-Derived 7-mer Peptides for the Interaction Between CFP-Nrf2 and YFP-Kelch**

| Peptide | Sequence       | FRET IC$_{50}$ ± SE (µM) | FP IC$_{50}$ ± SE (µM) |
|---------|----------------|---------------------------|-------------------------|
| 1       | Ac-DEETGEF-OH  | 3.34 ± 0.44               | 5.39 ± 0.58             |
| 2       | St-DEETGEF-OH  | 0.45 ± 0.05               | 0.18 ± 0.04             |
| 3       | Ac-DPETGEL-OH  | 0.33 ± 0.03               | 0.25 ± 0.04             |
| 4       | Ph-DPETGEL-OH  | 0.26 ± 0.03               | 0.16 ± 0.02             |
| 5       | St-DPETGEL-OH  | 0.12 ± 0.01               | 0.02 ± 0.003            |
| 6       | St-DEGETEF-OH  | 14.5 ± 1.60               | 11.8 ± 2.67             |
mM Tris-HCl pH 7.4 buffer containing, 0.5 mM DTT, 0.1 mM EDTA, and 5% v/v glycerol. Final concentrations of 0.05–0.80 μM or 0.01–0.50 μM YFP-Kelch or unconjugated YFP were added to a final concentration of 0.11 μM CFP-Nrf2 or CFP-Nrf2 mutant proteins. Fluorescence emission spectra of YFP-Kelch or unconjugated YFP and CFP-Nrf2 or CFP-Nrf2 mutant protein samples were recorded separately. Binding curves were fitted by nonlinear regression using SigmaPlot software (ligand binding, one site saturation) and Kd and Bmax values were determined.

**FRET competition assay.** Competition assays were performed using a Pherastar BMG Labtech microplate reader. In this format, a concentration of 0.11 μM CFP-Nrf2 and 0.20 μM YFP-Kelch were used. Assays were performed with increasing concentrations of peptide inhibitor (0.001–100 μM) at a final volume of 100 μL and a final DMSO concentration of 0.1% v/v in untreated black 96-well microtiter plates (Corning). All measurements were carried out in triplicate. Plates were read directly after mixing the components. FE50 were calculated using:

\[
FE = 1 - \frac{F_{d+a+inhibitor}}{F_{d+vehicle}}
\]

where \(d\) is the donor emission in the presence of the acceptor and \(a\) is the donor emission in the absence of the acceptor\(^{19}\) and vehicle is 0.1% DMSO. Percentage inhibition was determined using the calculated FE50. Inhibition curves were fitted to a standard four-parameter logistic function using SigmaPlot and IC50 values were determined.

**Acknowledgments**

The authors thank Dr Edwin Nkansah and Dr Andreia Guimaraes for providing plasmids and Hei Leung for her contribution to the recombinant protein production.

**References**

1. Wattenberg LW (1985) Chemoprevention of cancer. Cancer Res 45:1–8.
2. Manson MM, Gescher A, Hudson EA, Plummer SM, Squires MS, Prigent SA (2000) Blocking and suppressing mechanisms of chemoprevention by dietary constituents. Toxicol Lett 112-113:499–505.
3. Hayes JD, McMahon M, Chowdhry S, Dinkova-Kostova AT (2010) Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway. Antioxid Redox Signal 13:1713–1748.
4. Tong KI, Katoh Y, Kusunoki H, Itoh K, Tanaka T, Yamamoto M (2006) Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model. Mol Cell Biol 26:2887–2900.
5. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13:76–86.
6. Ahn YH, Hwang Y, Liu H, Wang XJ, Zhang Y, Stephenson KK, Borunina TN, Cole RN, Dinkova-Kostova AT, Talalay P, Cole PA (2010) Electrophilic tuning of the chemoprotective natural product sulforaphane. Proc Natl Acad Sci USA 107:9590–9595.
7. Lo SC, Li X, Henzl MT, Beamer LJ, Hannink M (2006) Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. EMBO J 25:3605–3617.
8. Dinkova-Kostova AT, Holtclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, Talalay P (2002) Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proc Natl Acad Sci USA 99:11908–11913.
9. Urano A, Motohashi H (2011) The Keap1-Nrf2 system as an in vivo sensor for electrophiles. Nitric Oxide 25:153–160.
10. Zhang DD, Hannink M (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 23:8137–8151.
11. Hancock R, Schaap M, Pfister H, Wells G (2013) Peptide inhibitors of the Keap1-Nrf2 protein-protein interaction with improved binding and cellular activity. Org Biomol Chem 11:3553–3557.
12. Magee S, Chen Y, Hu L (2012) Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents. Med Res Rev 32:687–726.
13. Hu L, Magee S, Chen L, Wang L, Lewis TA, Chen Y, Khodier C, Inoyama D, Beamer LJ, Emge TJ, Shen J, Kerrigan JE, Kong AN, Dandapani S, Palmer M, Schreiber SL, Munoz B (2013) Discovery of a small-molecule inhibitor and cellular probe of Keap1-Nrf2 protein-protein interaction. Bioorg Med Chem Lett 23:3039–3043.
14. Marcotte D, Zeng W, Hus JC, McKenzie A, Hession C, Jin P, Bergeron C, Lugovskoy A, Enyedi I, Cuervo H, Wang D, Atmane C, Roecdlin D, Vecchi M, Vivat V, Kraemer J, Winkler D, Hong V, Chao J, Lukashev M, Silvian L (2013) Small molecules inhibit the interaction of Nrf2 and the Keap1 Kelch domain through a non-covalent mechanism. Bioorg Med Chem 21:4011–4019.
15. Inoyama D, Chen Y, Huang X, Beamer LJ, Kong AN, Hu L (2012) Optimization of fluorescently labeled Nrf2 peptide probes and the development of a fluorescence polarization assay for the discovery of inhibitors of Keap1-Nrf2 interaction. J Biomol Screen 17:435–447.
16. Chen Y, Inoyama D, Kong AN, Beamer LJ, Hu L (2011) Kinetic analyses of Keap1-Nrf2 interaction and determination of the minimal Nrf2 peptide sequence required for Keap1 binding using surface plasmon resonance. Chem Biol Drug Des 78:1014–1021.
17. Shibata T, Ohta T, Tong KI, Kokubu A, Odogawa R, Tsuta K, Asamura H, Yamamoto M, Hirohashi S (2008) Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. Proc Natl Acad Sci USA 105:13568–13573.
18. Nkansah E, Shah R, Collie GW, Parkinson GN, Palmer J, Rahman KM, Bui TT, Drake AF, Husby J, Neidle S, Zinzailla G, Thurston DE, Wilderspin AF (2013) Observation of unphosphorylated STAT3 core protein binding.
to target dsDNA by PEMSA and X-ray crystallography. FEBS Lett 587:833–839.

19. Clegg RM. Förster resonance energy transfer—FRET what is it, why do it, and how it’s done. In: Gadella TWJ, Ed. (2009) Laboratory techniques in biochemistry and molecular biology. Elsevier B.V., Urbana: Illinois, p 38.

20. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 25:1605–1612.

21. Hancock R, Bertrand HC, Tsujita T, Naz S, El-Bakry A, Laoruchupong J, Hayes JD, Wells G (2012) Peptide inhibitors of the Keap1-Nrf2 protein-protein interaction. Free Radic Biol Med 52:444–451.

22. Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY (1995) Understanding, improving and using green fluorescent proteins. Trends Biochem Sci 20:448–455.

23. Tsien RY (1998) The green fluorescent protein. Annu Rev Biochem 67:509–544.

24. Pollok BA, Heim R (1999) Using GFP in FRET-based applications. Trends Cell Biol 9:57–60.

25. Martin SF, Tatham MH, Hay RT, Samuel ID (2008) Quantitative analysis of multi-protein interactions using FRET: application to the SUMO pathway. Prot Sci 17:777–784.

26. Piston DW, Kremers GJ (2007) Fluorescent protein FRET: the good, the bad and the ugly. Trends in Biochem Sci 32:407–414.

27. Liang Y (2008) Applications of isothermal titration calorimetry in protein science. Acta Biochim Biophys Sin (Shanghai) 40:565–576.

28. Zhou X, Sun Q, Kini RM, Sivaraman J (2008) A universal method for fishing target proteins from mixtures of biomolecules using isothermal titration calorimetry. Prot Sci 17:1798–1804.

29. Ladbury JE, Klebe G, Freire E (2010) Adding calorimetric data to decision making in lead discovery: a hot tip. Nat Rev Drug Discov 9:23–27.

30. Neumann T, Junker HD, Schmidt K, Sekul R (2007) SPR-based fragment screening: advantages and applications. Curr Top Med Chem 7:1630–1642.

31. Inoyama D, Chen Y, Huang XY, Beamer LJ, Kong ANT, Hu LQ (2012) Optimization of fluorescently labeled Nrf2 peptide probes and the development of a fluorescence polarization assay for the discovery of inhibitors of Keap1-Nrf2 interaction. J Biomol Screen 17:435–447.

32. Song Y, Madahar V, Liao J (2011) Development of FRET assay into quantitative and high-throughput screening technology platforms for protein-protein interactions. Ann Biomed Eng 39:1224–1234.

33. Wang XJ, Hayes JD, Wolf CR (2006) Generation of a stable antioxidant response element-driven reporter gene cell line and its use to show redox-dependent activation of nrf2 by cancer chemotherapeutic agents. Cancer Res 66:10983–10994.

34. Prochaska HJ, Santamaria AB (1988) Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. Anal Biochem 169:328–336.

35. Baird L, Lleres D, Swift S, Dinkova-Kostova AT (2013) Regulatory flexibility in the Nrf2-mediated stress response is conferred by conformational cycling of the Keap1-Nrf2 protein complex. Proc Natl Acad Sci USA 110:15259–15264.