Visualisation of Endogenous ERK1/2 in Cells with a Bioorthogonal Covalent Probe

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Abstract

The RAS-RAF-MEK-ERK pathway has been intensively studied in oncology with RAS known to be mutated in ~30% of all human cancers. The recent emergence of ERK1/2 inhibitors and their ongoing clinical investigation demands a better understanding of ERK1/2 behaviour following small molecule inhibition. Although fluorescent fusion proteins and fluorescent antibodies are well-established methods to visualise proteins, we show that ERK1/2 can be visualised *via* a less invasive approach based on a two-step process using Inverse Electron Demand Diels-Alder cycloaddition. Our previously reported TCO-tagged covalent ERK1/2 inhibitor was used in a series of imaging experiments following a click reaction with a tetrazine-tagged fluorescent dye. Although limitations were encountered with this approach, endogenous ERK1/2 was successfully imaged in cells and 'on target' staining was confirmed by overexpressing DUSP5, a nuclear ERK1/2 phosphatase which anchors ERK1/2 in the nucleus.

Introduction

The RAS-RAF-MEK-ERK signalling pathway is activated by extracellular stimuli to govern cell proliferation, differentiation and survival.[1] RAS is a small GTPase that is mutated in ~30% of all human cancers causing persistent activation of the signalling cascade.[2] This has motivated significant investment in the development of inhibitors of the RAS-RAF-MEK-ERK pathway as therapeutic agents.[3, 4] In recent years, small molecule inhibitors of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) have been developed[5-9] with several currently undergoing clinical investigation. ERK1/2 are highly homologous serine-threonine kinases found primarily in the cytoplasm of resting cells[1] that become activated by dual phosphorylation of their activation loop, catalysed by MEK1/2.[10] Upon activation, ERK1/2 are released from MEK1/2 and can translocate to the nucleus where they regulate numerous substrates including transcription factors.[11, 12]

Given the importance of ERK1/2 in oncology, a deeper understanding of their spatiotemporal dynamics and activity upon small molecule inhibition would be beneficial. ERK2-GFP fusion proteins have been used to study the localisation of ERK2[13], whilst a FRET probe, in which ERK2 was modified with the addition of YFP and CFP has been used to study its activation.[14] Although the use of fluorescent proteins (FPs) is an attractive strategy, the FP-fusion protein approach suffers from limitations such as overexpression of the FP fusion protein compared to the endogenous proteins that can result in changes in localisation,[15, 16] the risk of undesired oligomerisation[17] and the long maturation time.[17] The steric hindrance imposed by the FPs, and also by fluorescent antibodies, needs to be considered when interpreting observations as it is likely to impact on protein-protein interactions and alter the protein's functions. A less invasive method to visualise endogenous proteins in cells, which has yet to be explored for ERK1/2, is the use of a fluorescent chemical probe. This strategy relies on the modification of a known ligand by the direct addition of a fluorophore or a clickable handle for the indirect introduction

of a fluorescent group *via* a bioorthogonal reaction. The direct attachment of a fluorophore to a ligand generates a high molecular weight probe which commonly leads to problems with cell permeation. As such, a two-step labelling approach using click chemistry is as an attractive alternative.

Among the bioorthogonal reactions available in the literature,[18, 19] the Inverse Electron Demand Diels-Alder (IEDDA) cycloaddition (with a rate constant of $k_2 \sim 10^2$-10^6 $M^{-1}.s^{-1}$)[20] offers the possibility to study fast biological processes as well as low abundance proteins[19] with a high signal-to-noise ratio.[21] No additional reagents are required other than the two reactive partners, a tetrazine and a strained alkene such as norbornene[22] or *trans*-cyclooctene (TCO).[21] The IEDDA cycloaddition has been successfully used in fluorescence imaging to study the localisation of a number of proteins including AKA,[23] PLK1[24] and PARP1.[25, 26] Here, we investigated the ability of our previously published covalent ERK1/2 TCO probe[27] to image endogenous ERK1/2 in cells after a click reaction with a fluorescent tetrazine dye.

Results and Discussion

Pharmacological profile of Probe 1

We previously reported the design and synthesis of a covalent ERK1/2 TCO probe (Probe 1, **Fig. 1A**) for which selectivity profile and target engagement were studied using in-gel fluorescence.[27] The results obtained suggested that Probe 1 might be a promising tool compound for ERK1/2 imaging, so we investigated its pharmacological profile in order to determine the optimum concentration and incubation time for imaging experiments. SW620 cells, a colon carcinoma cell line with activated ERK pathway signalling, were treated with Probe 1 or the untagged parent inhibitor, compound 2 (**Fig. 1A**), for 3 hours at different concentrations. Following cell lysis, the expression of downstream and upstream substrates was studied by immunoblotting. Probe 1 was found to be less potent at matched concentrations

with compound 2 (**Fig. 1B** and **Supplementary Fig. 1**), as measured by the reduction in downstream ERK1/2 targets such as p-RSK, FRA1 and the stabilisation of BIM. Similarly, compound 2 showed a greater increase in p-MEK1/2 compared to Probe 1 as a result of the loss of negative feedback loops upon ERK1/2 inhibition. From the selectivity studies conducted on Probe 1 by in-gel fluorescence (**Supplementary Fig. 2**), a good compromise between on- and off-target activities was found at 300 nM. Next, the time course of covalent binding of Probe 1 to ERK1/2 was studied. SW620 cells were treated with Probe 1 (300 nM) and lysed at different time points. Subsequently, the lysates were treated with Sulfo-Cy5-Tz (10 µM, 6 h, 4 °C) and analysed by in-gel fluorescence (**Fig. 1C** and **1D, Supplementary Fig. 3**). Quantitation of the intensity of the fluorescent band at ~42 kDa indicated complete formation of the ERK-Probe 1 adduct after 8 hours of incubation. We previously reported the *in vitro* study of the covalent binding of Probe 1 to ERK2 by mass spectrometry, showing >90% of recombinant ERK2 was covalently bound to Probe 1 after 10 min of incubation (ERK2: Probe 1 1:10). The difference between the rates of adduct formation observed in the enzymatic and cellular studies may arise from differences in concentrations of ERK2 and Probe 1 in cells, including non-specific and off-target binding, which likely reduce the free concentration of Probe 1, as well as competition with ATP in cells. As a result of these observations, an incubation time of 3 hours was selected for the imaging experiments as it was considered long enough for the covalent binding to occur but short enough to limit cell death due to the cytotoxicity of the compound.

ERK1/2 imaging in cells

Highly fluorescent background is a major limiting factor in the quality of fluorescence imaging. For our imaging experiments we compared two tetrazine-containing dyes suitable for click combination with Probe 1: the permanently fluorescent dye FAM-Tz; and a recently developed turn-on dye, BODIPY-Tz, in which the fluorescence is quenched by

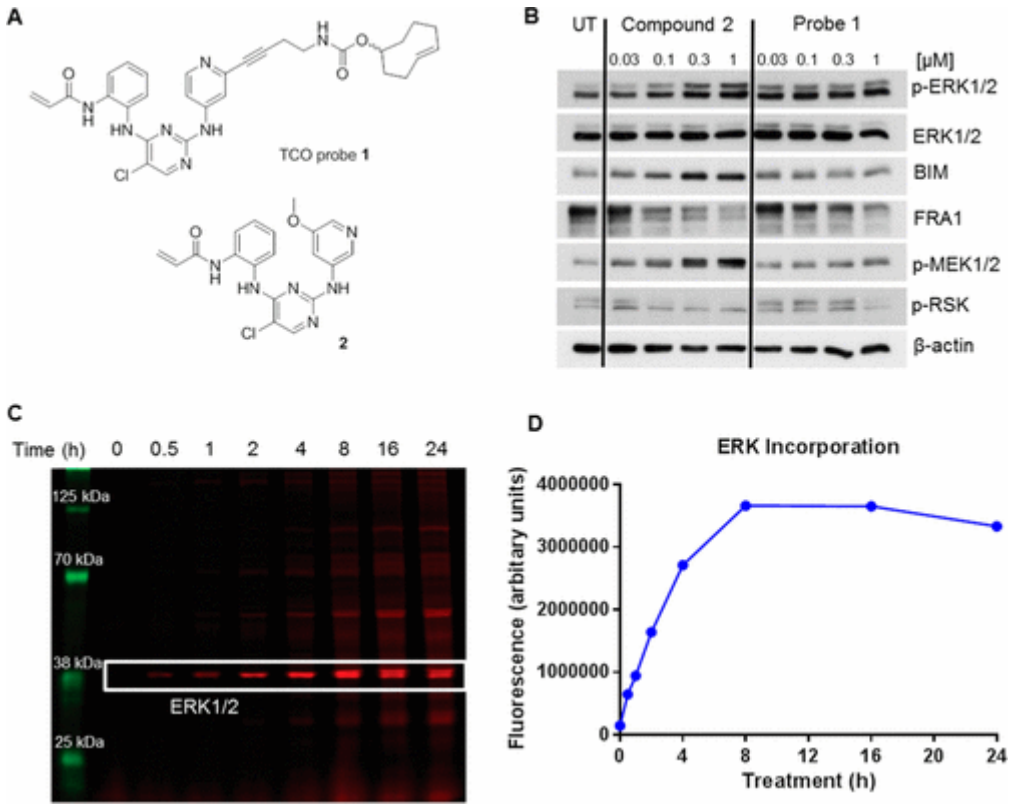

Figure 1. A. Structures of Probe 1 and compound 2. B. Immunoblots for ERK1/2, its downstream substrates and activating kinases (MEK1/2) showing the pharmacological profiles of compound 2 and Probe 1 at various concentrations. SW620 cells were treated with compound 2 or probe 1 or no drug (UT) for 3 hours. C. In-gel fluorescence used to quantify the formation of the ERK-Probe 1 adduct. SW620 cells were treated with Probe 1 (300 nM) for the indicated times, lysed and incubated with Sulfo-Cy5-Tz dye (10 μM) for 6 hours at 4 °C then separated by SDS-PAGE. D. Plot showing fluorescence from the formation of the ERK-Probe 1 adduct against time calculated by densitometry from the in-gel fluorescence experiment. Data shown is representative of at least 3 biological replicate experiments.

through bond energy transfer (TBET) with a tetrazine ring: upon click reaction with a TCO ligand, the tetrazine ring is lost and the fluorescence of the dye is released.[28] To ensure that the click reactions between Probe 1 and the tetrazine dyes proceed in high yield, the cycloadditions were studied by LC-MS in the absence of cells (**Supplementary Fig. 4** and **5**). Probe 1 and

either BODIPY-Tz or FAM-Tz were mixed in MeOH. The reactions were over 90% complete with either dye after 10 min. This allowed us to use the Tz-dye only in a 3-fold excess in the following experiments, limiting residual fluorescence due to unwashed, excess dye. Indeed, even though the cells are washed to remove unreacted dyes in imaging experiments, it is often difficult to wash out all the excess of dye.[29]

To assess the suitability of Probe 1 for imaging, its fluorescent labelling by Tz-dyes was studied in cells (**Fig. 2A**). SW620 cells were treated with Probe 1 at different concentrations for 3 hours followed by FAM-Tz (1 µM, 15 min) post fixation of the cells (**Supplementary Fig. 6**). When used at 300 nM, Probe 1 gave a fluorescent signal well above that of background, which was largely cytoplasmic, focussed around the perinuclear region, leaving a nuclear hole in the dye channel (**Fig. 2B**). SW620 cells were also incubated with the Tz-dye alone (FAM-Tz 1 µM, 15 min post fixation (**Fig. 2B**) or BODIPY-Tz alone (1 µM, 10 min) post fixation (**Supplementary Fig. 7**)) to determine the fluorescent background associated with the dye. In our experiments, low background fluorescence was observed for both BODIPY-Tz and FAM-Tz, such that signal due to Probe 1 was well above that of background staining.

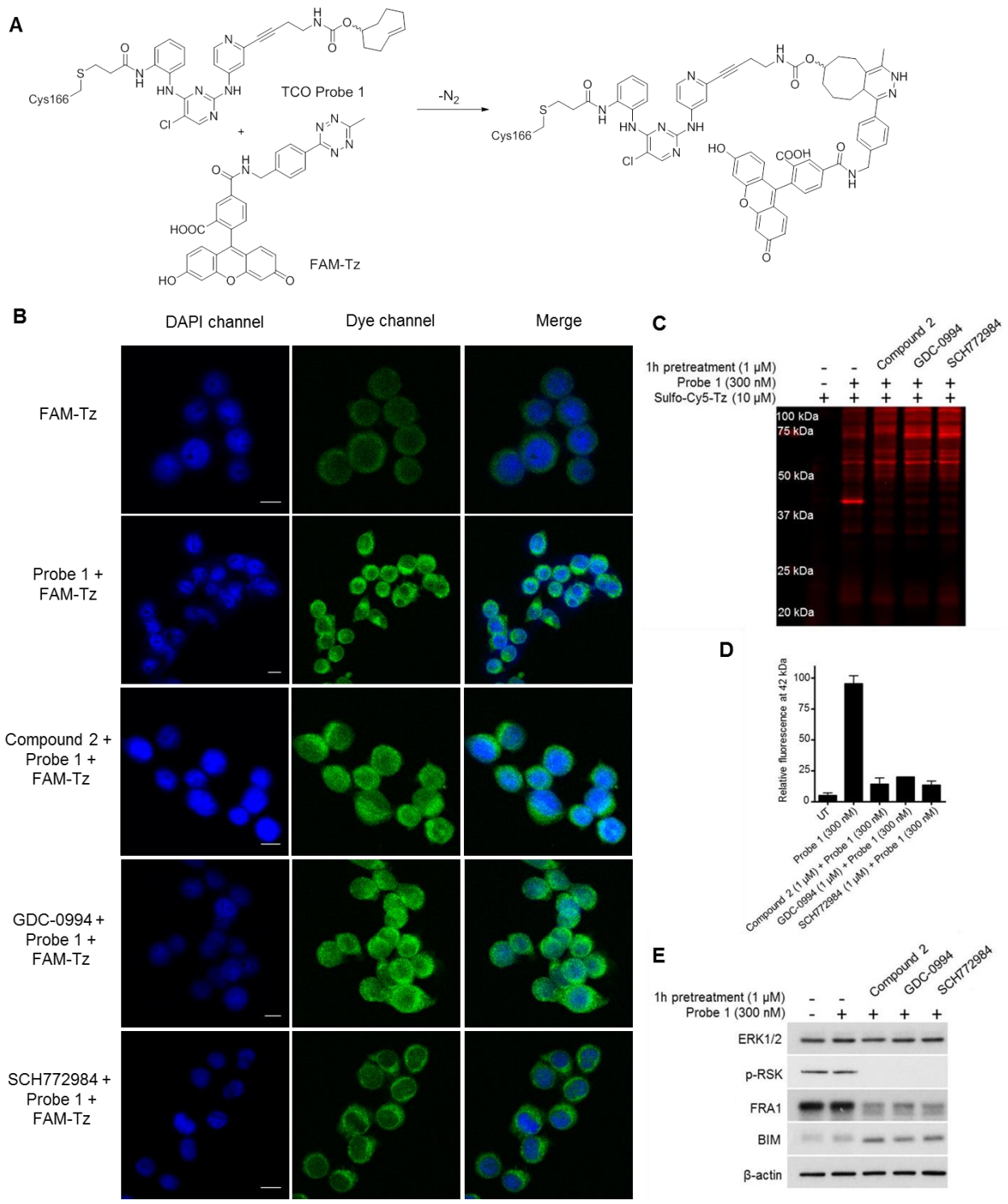

Figure 2. A. 'Click' reaction between Probe 1 and FAM-Tz. B. Images showing fixed SW620 cells after treatment with FAM-Tz (1 μM, 15 min) alone, with Probe 1 (300 nM, 3 hours) and FAM-Tz (1 μM, 15 min), and when the cells were pre-treated with compound 2, GDC-0994 or SCH772984 (1 μM, 4 hours) followed by Probe 1 (300 nM, 3 hours) and FAM-Tz (1 μM, 15 min). The nuclei are shown in blue (DAPI staining) and the fluorescence from the click reaction is shown in green (dye channel) and

a merged image of the two channels (merge). The white bar represents 10 µm. C. In-gel fluorescence showing disappearance of the fluorescent band at 42 kDa (the molecular weight of ERK2) by competition with Compound 2, GDC-0994 or SCH772984. SW620 cells were pre-treated with compound 2, GDC-0994 or SCH772984 (1 µM, 4 hours) followed by Probe 1 (300 nM, 3 hours) and the cleared cell lysates were then treated with Sulfo-Cy5-Tz dye (10 µM, 6 hours, 4 °C) then separated by SDS-PAGE. D. Bar chart quantifying the decrease in fluorescence of the band at 42 kDa, following the pre-treatment of SW620 cells with compound 2, GDC-0994 or SCH772984. E. The immunoblotting analysis showed the disappearance of the p-RSK and FRA1 bands and stabilisation of BIM when cells were pre-treated with the ERK inhibitors, confirming the inhibition of ERK1/2. Data shown is representative of at least 2 biological replicate experiments.

We then carried out a series of control experiments to validate the fluorescent images observed with Probe 1 and Tz-dyes. We first performed competition experiments in which SW620 cells were pre-treated with the untagged covalent inhibitor Compound 2 or the structurally distinct non-covalent ERK inhibitors GDC-0994 or SCH772984 (1 µM, 1 h) before addition of Probe 1 (300 nM, 3 h) (**Fig. 2B**, **Supplementary Fig. 7**). The Tz-dye, FAM-Tz or BODIPY-Tz (1 µM, 10 or 15 min), was added either following or prior to fixation and permeabilisation of cells. By pre-incubating the cells with untagged inhibitors, the fluorescence signal was expected to decrease due to a reduction in ERK1/2 available for binding with Probe 1. However, with both dyes, we did not observe a significant change in fluorescence (**Fig. 2B**, **Supplementary Fig. 7**).

In-gel fluorescence experiments applying the same conditions as used for imaging, followed by cell lysis and reaction with Sulfo-Cy5-Tz dye (10 µM, 6 hours, 4 °C), confirmed that compound 2, as well as the other structurally unrelated ERK inhibitors, successfully diminished the fluorescent labelling of ERK1/2 by Probe 1, as evidenced by a decrease in fluorescence of the band at ~42 kDa (**Fig. 2C**, **2D** and **Supplementary Fig. 8**). In an effort to confirm that the

fluorescence seen within the imaging experiments was due to ERK1/2 localisation, wild type (WT) and ERK2 knock-out (KO) MEFs (mouse embryonic fibroblasts) were utilised, as a change in signal localisation and strength would be expected in the ERK2 KO cells. The cells were treated with Probe 1 (300 nM, 3 h) followed by BODIPY-Tz (1 μM, 15 min). Surprisingly, the fluorescence signal was still present and with a similar staining pattern in ERK2 KO and WT MEFs (**Fig. 3A**). To understand better our observations, we treated both WT and ERK2 KO MEFs with Probe 1 (300 nM, 3 h) and following cell lysis and treatment with Sulfo-Cy5-Tz dye (1 μM, 6 hours, 4 °C), the lysates were studied by in-gel fluorescence and immunoblotting (**Fig. 3B** and **3C, Supplementary Fig. 9**). As expected, a reduction of fluorescence of the band at ~42 kDa was observed in ERK2 KO cells compared with WT cells (**Fig. 3D**).

Taken together, these findings suggested that the image observed with Probe 1 corresponded to the sum of its labelling of ERK1/2 together with imaging of additional 'off-target' proteins or non-specific binding events. [30] Indeed, unless a probe possesses exquisite selectivity for its target, overexpression may be required in order to achieve a strong enough signal compared with the image of off-targets; for example, recent work describing the imaging of Dasatinib targets using a TCO analogue of Dasatinib in cells required artificial over-expression of the proteins of interest in specific localisations in the cell.[31]

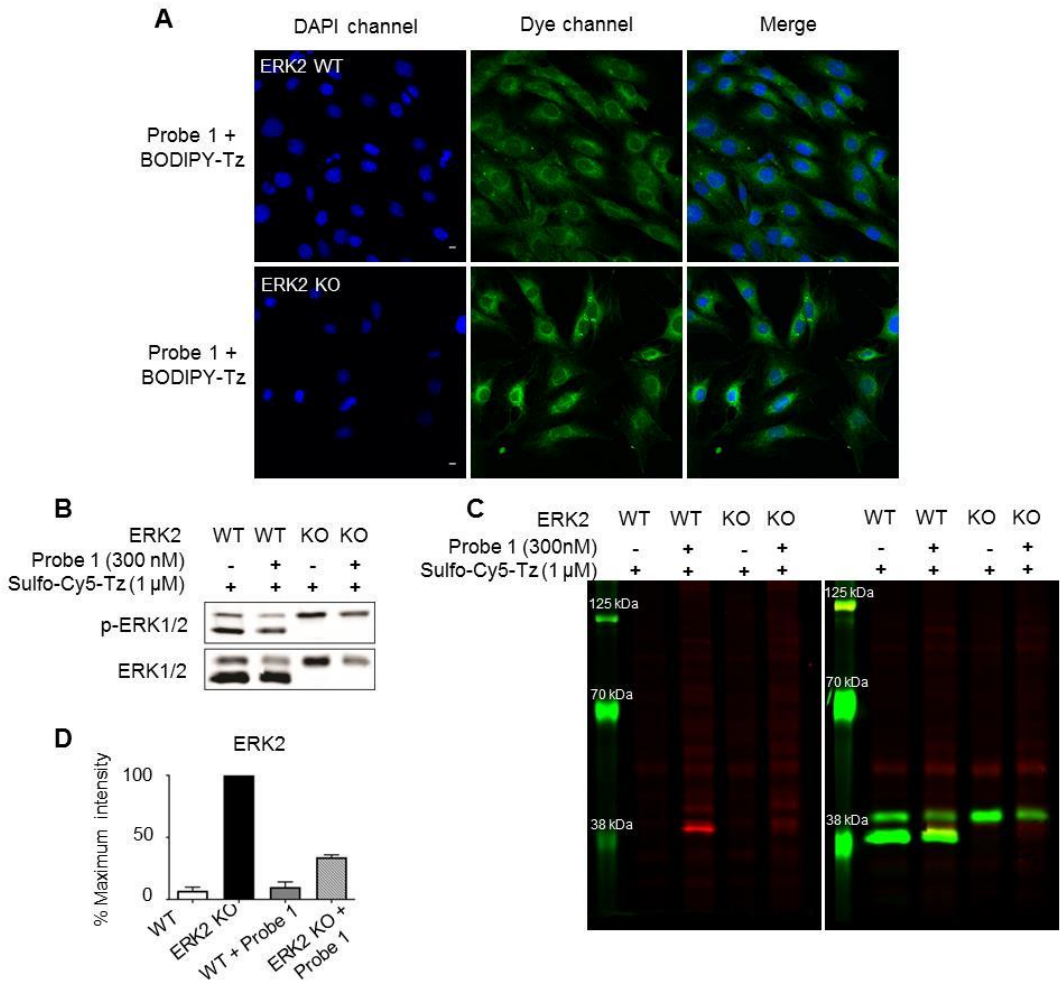

Figure 3. A. Images showing WT MEF and ERK2 KO MEF cells after click reaction between Probe 1 (300 nM, 3 h) and BODIPY-Tz (1 µM, 15 min). The nuclei are shown in blue (DAPI staining) and the fluorescence from the click reaction is shown in green (dye channel) and a merged image of the two channels (merge). The white bar represents 10 µm. B. Immunoblotting showing the expression of ERK1/2 and phospho-ERK1/2 in WT MEF and ERK2 KO MEF cells. C. In-gel fluorescence illustrating on- and off-targets of Probe 1 (300 nM, 3 h). (Left panel) A decrease in fluorescence of the band at 42 kDa (ERK2) in ERK2 KO cells was observed. (Right panel) ERK1/2 antibody signal (green) confirmed the main band at 42 kDa as ERK2 and the secondary band as ERK1. D. Bar charts showing the relative fluorescence of the band corresponding to ERK2, from the in-gel fluorescence experiment. Data shown are mean, normalised fluorescence values +/-SD, of two biological replicates.

To overcome the issue of the specific ERK1/2 signal being masked by non-specific fluorescence, we decided to study the ability of Probe 1 to image endogenous ERK1/2 under conditions in which its localisation is modified through overexpression of DUSP5, a nuclear ERK1/2-specific phosphatase which dephosphorylates and anchors ERK1/2 in the nucleus.[32] To ensure that any change in signal due to Probe 1 was a result of ERK1/2 re-localisation, we also used a DUSP5 construct containing a mutation within its kinase-interacting motif (KIM), to prevent its association with ERK1/2. HEK293 cells, a human embryonic kidney cell line, were transfected with a pCMV3.1 vector containing either the DUSP5-MYC or DUSP5-KIM-MYC construct for 24 hours. Cells were then incubated with Probe 1 (300 nM, 3 h) followed by cell fixation, permeabilisation and treatment with FAM-Tz (1 μM, 10 min). In cells expressing DUSP5 (MYC antibody signal, red, **Fig. 4**), ERK1/2 was found to re-localise to the nucleus (ERK1/2 antibody signal, grey, **Fig. 4**) and similarly, the signal due to Probe 1 also moved to the nucleus (green, **Fig. 4**), whilst cells not expressing the construct, or those that expressed DUSP5-KIM, showed no change in ERK1/2 localisation (ERK1/2 antibody and Probe 1 signals, **Fig. 4**). An excellent correlation was observed between the fluorescence signal from the click reaction between Probe 1 and FAM-Tz (green, **Fig. 4**) with the MYC and ERK1/2 antibodies signals (red and grey, respectively, **Fig. 4**) confirming the potential of Probe 1 to image endogenous ERK1/2 in cells. We quantified the changes in Probe 1 localisation in cells expressing DUSP5 versus DUSP5-KIM. Within each experiment, cells were binned based on whether they expressed either DUSP5 or DUSP5-KIM, or did not express the construct. The average intensity of nuclear fluorescence was then assessed in the DUSP5 or DUSP5-KIM positive cells versus negative cells (**Supplementary Fig. 10**). DUSP5-positive cells showed a significant increase in their average nuclear staining intensity compared to DUSP5-negative cells ($p=0.011$) whilst DUSP5-KIM cells showed no significant increase in average nuclear fluorescence ($p=0.985$).

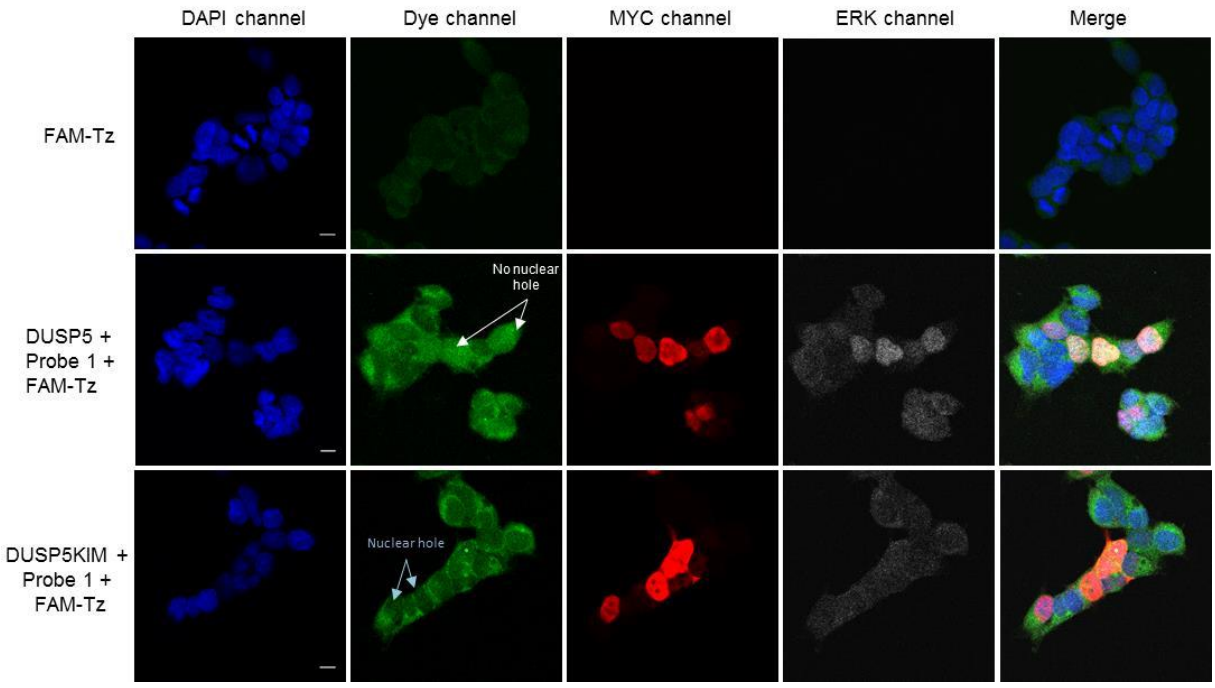

Figure 4. Images showing fixed HEK293 cells that were transfected with DUSP5 or DUSP5-KIM constructs and treated with Probe 1 (300 nM, 3 h) and FAM-Tz (1 µM, 10 min). With DUSP5 overexpressed, the fluorescence from the click reaction between Probe 1 and FAM-Tz was found spread in the nucleus and cytoplasm. When DUSP5-KIM was expressed, the fluorescence was localised in the cytoplasm creating nuclear holes. The nuclei are shown in blue (DAPI staining). The fluorescence from the click reaction between Probe 1 and FAM-Tz is shown in green (Dye channel). DUSP5-MYC is shown in red (MYC channel) and ERK1/2 is shown in grey (ERK1/2 channel). The different staining were overlapped (merge) demonstrating good correlation between DUSP5 expression and homogenous fluorescence staining in the dye channel. The white bar represents 10 µm. Data shown is representative of 4 biological replicate experiments.

Conclusions

In this work we have studied the suitability of a covalent ERK1/2 probe for cellular imaging experiments. The results indicated that although ERK1/2 is labelled and imaged by Probe 1, the image of ERK1/2 is masked by concurrent off-target staining. However, modulation of ERK1/2 localisation by overexpression of DUSP5 allowed a clear signal due to ERK1/2

labelling to be observed in the nucleus. Thus Probe 1 provides a system by which endogenous ERK1/2 can be observed without overexpression. These experiments further demonstrate the versatility of a bioorthogonally-tagged probe compound such as Probe 1 across a range of chemical biology applications, adding cell imaging to the previously reported studies using Probe 1 for in-gel selectivity profiling and for protein degradation upon click reaction with a tetrazine thalidomide reagent.[33]

Experimental Procedures

Materials. FAM-Tz and Sulfo-Cy5-Tz were purchased from JenaBioscience and was used as received. BODIPY-Tz[28] and Probe 1[27] were synthesised following the literature precedents. SW620 cells were bought from A.T.C.C. WT and ERK2 KO MEFs were generated as described.[34] HEK293 cells were kindly provided by Dr Nicholas Ktistakis (Babraham Institute). DUSP5 and DUSP5KIM vectors were kindly provided by Dr Christopher J. Caunt (University of Bath). Antibodies p-ERK1/2 (9101), ERK1/2 (4695S), p-MEK1/2 (9121), MEK1/2 (9122), p-p90RSK T359 (8753), p-90RSK (9355) and p-RSK S380 (9341) were purchased from Cell Signaling Technology, MYC (9E10, sc40) and FRA1 (N-17, sc183) were purchased from Santa Cruz Biotechnology, BIM (AB17003) was purchased from Merck Millipore and β-actin (A5441) from Sigma Aldrich. GDC-0994 and SCH772984 were purchased from SelleckChem (S7554 and S7101 respectively).

Methods. See Supporting Information.

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Supporting Information

Supplementary Figures 1-10; Experimental procedures for LC-MS, cell culture, in-gel fluorescence, cell proliferation assay, immunoblotting, transfection, fluorescence imaging and immunofluorescence, and ERK1/2 inhibitor competition experiments.

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Abbreviations

TCO: *trans*-cyclooctene

Tz: Tetrazine

FP: Fluorescent Protein

Sulfo-Cy5-Tz: 6-Methyl-tetrazine SulfoCy5

FAM-Tz: (4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine-5-Fluorescein

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