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A method for visualizing fluorescence of flavonoid therapeutics *in vivo* in the model eukaryote *Dictyostelium discoideum*

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ABSTRACT

Naturstoff reagent A (diphenylboric acid 2-aminoethyl ester [DPBA]) has been used historically in plant science to observe polyphenolic pigments, such as flavonoids, whose fluorescence requires enhancement to be visible by microscopy. Flavonoids are common dietary constituents and are the focus of considerable attention because of their potential as novel therapies for numerous diseases. The molecular basis of therapeutic activity is only gradually being established, and one strand of such research is making use of the social amoeba *Dictyostelium discoideum*. We extended the application of DPBA to flavonoid imaging in these preclinical studies, and report the first method for use of DPBA in this eukaryotic model microbe and its applicability alongside subcellular markers. This *in vivo* fluorescence imaging provided a useful adjunct to parallel chemical and genetic studies.

METHOD SUMMARY

Flavonoids at physiological concentrations emit only low levels of fluorescence, which are not visible by microscopy *in situ*. A dye that has been used to enhance fluorescence of polyphenolic pigments in plant tissues was found to be effective in studies of flavonoid therapeutics in a model eukaryotic microbe, *Dictyostelium discoideum*. Use of a simple buffer and fixative on treated cells allows flavonoid transport and localization to be monitored *in vivo*, using epifluorescence and confocal microscopy.

KEYWORDS

Dictyostelium • flavonoid • fluorescence • microscopy

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INTRODUCTION

Flavonoids constitute a large family of polyphenolic compounds that have been widely suggested to endow health benefits, ranging from anticancer, anti-inflammatory, antiviral and anti-allergic activity, through to treatment of diabetes and Alzheimer's disease [1–3]. Flavonoids are not synthesized by animals or their gut microbiota but are abundant in our diet because they are secondary metabolites of plants. They act as pigments and bitter flavours, attracting pollinators or protecting against UV and herbivory, but can also modulate plant development and root–microbial symbioses [4–6]. The anti-oxidant activity shared by many of these compounds has been hypothesized to underlie purported therapeutic benefits, and numerous studies do demonstrate links between health outcomes and flavonoids. The literature, however, does not describe well the specific transport, biological activity and chemical fate upon ingestion of individual compounds. These are subject to compound size, configuration, lipophilicity and solubility, and flavonoids possess diverse chemical subgroups and modifications, the latter including hydroxylation, glycosylation, methylation and polymerization (Figure 1) [7]. For example, such compound specificity was described previously for the activities of plant polyphenols: only a subset of flavonoids affects the transport of the plant hormone auxin, and thus plant development [6,8,9]. The biomedical literature has recently begun to follow suit, with reports of particular polyphenolic secondary metabolites acting via individual protein targets and reaching distinct subcellular compartments [10,11]. There is also limited evidence for some pathways of flavonoid metabolism in humans, namely glucuronidation, *o*-methylation, and addition of glutathione, depending on where the compounds are absorbed [12]. Of note therapeutically, aglycones have been reported to be readily absorbed whereas glycosides must be transformed into the aglycone form [13,14]. Flavonoids, however, are most commonly found as glycosides in plants [15], so there are questions over transport of compounds even when robustly shown to have useful therapeutic activity.

Examples of evidence for activity of kaempferol (3,4′,5,7-tetrahydroxyflavone) were the inhibition of growth of lung cancer lines [16] and induction of cancer-cell apoptosis [15]. Naringenin (4′,5,7-trihydroxyflavanone) reduced cyst formation in both animal and *in vitro* studies of polycystic kidney disease (PKD): indeed its action via the PKD2 protein was first demonstrated in the model microbe *Dictyostelium* [11]. Furthermore, naringenin, kaempferol and quercetin (3,3′,4′,5,7-pentahydroxyflavone) have been reported to inhibit phosphoinositide 3-kinase, nuclear factor B pathway and kinases involved in pro-apoptic signaling in cancer cells [17,18].

These polyphenolic compounds can be quantified in tissue extracts by UV-visible spectroscopy, HPLC, mass spectrometry methods and NMR [19,20]. Flavonoids exhibit only low levels of fluorescence, however, and cannot be viewed at physiologically relevant concentrations *in vivo*. A method was therefore developed several decades ago for visualizing them in plant tissues using the fluorescent probe Naturstoff reagent A (diphenylboric acid 2-aminoethyl ester [DPBA]). The specificity of the probe for flavonoids was demonstrated in plants including *Arabidopsis thaliana* [21–23] and it has, infrequently, been applied in human *in vitro* cell cultures. For example, apigenin (4′,5,7-trihydroxyflavone) was visualized in mitochondria of monocytic leukemia (THP-1) cells [24]; quercetin and kaempferol in the nucleus of umbilical endothelial cells; and quercetin at the cell membrane in epithelial colorectal adenocarcinoma cells [7,25,26]. ▶

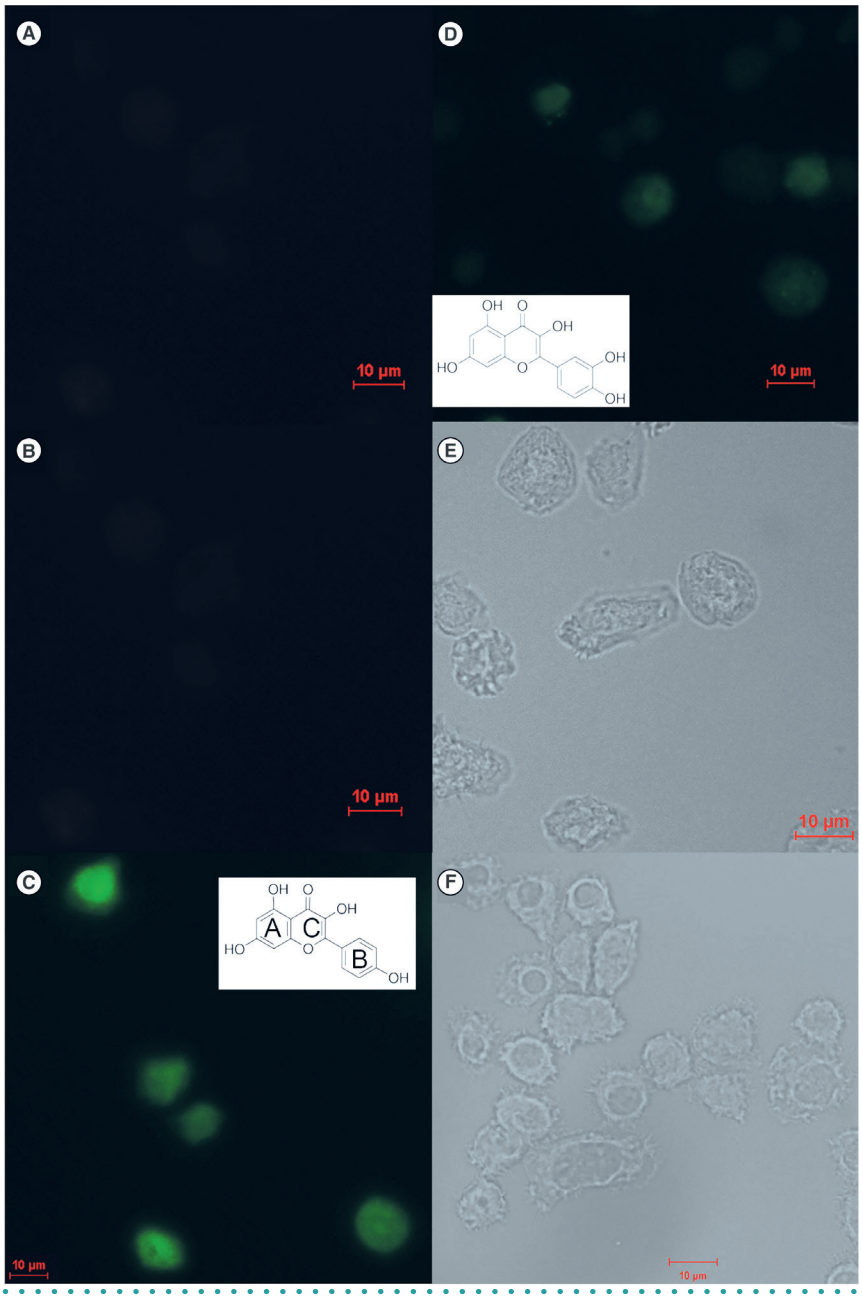

Figure 1. Epifluorescence microscopy testing for autofluorescence. In **(A)** untreated *Dictyostelium* cells vs **(B)** cells treated with DPBA vs cells exposed to 50 µM flavonoid treatments for 30 min (**C**, kaempferol; **D**, quercetin). Bright field **(E)** DPBA- and **(F)** DMSO-treated *Dictyostelium* cells. Images acquired using Nikon Eclipse 90i, Ex 470nm – Em 525 x60 lens.
DBPA: Diphenylboric acid 2-aminoethyl ester; DMSO: Dimethyl sulfoxide.

The mechanism by which the fluorescent probe binds to polyphenolics is not well described: it was proposed to form a spontaneous complex with flavonoids, resulting in an increase in fluorescence [27], but this is not the case for all members of the family. The use of DPBA was recorded by the German researcher Neu in 1961 [28], who described its synthesis by modifying Schiff's reagent with a nitrogen donor and two aromatic aldehydes. Neu suggested various complex formations with a range of pyridine compounds, and areas of complexation, and hypothesized that a minimum of one hydroxyl group was required for conjugation [28]. Two decades later, Brasseur and Angenot [29] showed that a minimum of two hydroxyl groups (Figure 1) were required for the flavonoid–DPBA conjugate to form. They also recorded the colour of fluorescence emission from a range of polyphenolic compounds under UV excitation [29]. In 2011, Matteini *et al.* [30] described various possibilities for complex formation between DPBA and rutin [3,3′,4′,5,7-pentahydroxyflavone 3-(O-rhamnosylglucoside)] and hypothesized that DPBA could conjugate at two sites of the flavonoid backbone, either between the 5-hydroxy-4-keto sites of the A–C ring, or the 3–4-*o*-diphenolic site of the B ring (Figure 1).

We explored further the utility of DPBA, with a range of plant or microbial metabolites whose biological activity was under investigation in a biomedical model microbe. The social amoeba *Dictyostelium discoideum* is a eukaryotic model organism whose life cycle includes phagocytosis of bacterial prey, before signaling and aggregation of motile cells leads to formation of a multicellular 'slug' with differentiated cell types [31]. It is an ideal biomedical model for various aspect of cell biology [32,33], and *D. discoideum* is also no stranger to flavonoid research. For example, a *Dictyostelium* mutant library was employed in the studies of naringenin, which identified the PKD2 cation-channel target [11] and of curcumin [34], and the amoeba was also employed to explore the chemotactic effect of bitter tastants [35]. Given the evidence for differential uptake of flavonoids, including long-distance transport [36], for involvement of specific transporter proteins [9,37,38] and the possibility of efflux (unpublished data), there is a need to observe localization of flavonoid-treatment and target *in vivo*. We found that differential uptake and targeting of therapeutically relevant flavonoids in the model amoeba could be visualized, and flavonoid localization could be imaged along with other cell markers, using the DPBA probe.

MATERIALS & METHODS

D. discoideum cells were grown axenically in HL5 medium (Formedium, Hunstanton, UK) at 22°C to a density of 10^6 cells/ml. Cells were washed twice by pelleting (500 × g, 4 min) and resuspended in potassium dibasic (KK2) buffer. Cells were then pelleted and resuspended in LoFlo broth (Formedium) at 5×10^5 cells/ml and re-incubated (22°C 180 rpm) for 24 h. After incubation, cells were transferred to a Petri dish containing sterile coverslips: 15 ml of cells at a final density of 5×10^6 cells/ml in LoFlo were allowed to settle onto the coverslips for 30 min.

For treatment with flavonoids, the appropriate molarity of the compound of interest was prepared using a maximum of 0.1% dimethyl sulfoxide (DMSO) in deionized water (no flavonoid, versus final concentrations of DMSO, were 25 μM, 0.025%; 50 μM, 0.05%; 100 μM, 0.1%). The selected treatment was added to the 15 ml LoFlo broth in a Petri dish, into which the coverslips and attached cells were transferred. Incubation for 30 min (or 0, 20, 40 or 60 min for time-course assay) at 22°C was followed with rinsing, by dipping coverslips in 0.1 M potassium phosphate buffer (PPB) pH6.8, then drying excess liquid from the glass with filter paper (Whatmann, Maidstone, UK), by capillary action.

For imaging, 0.1% w/v DPBA solution in ethanol was pipetted onto treated, adhered cells on coverslips, and allowed to stain for 5 min. DPBA was rinsed from coverslips by dipping into 0.1 M PPB once more, and blotting dry with filter discs again. Where required, cells were treated with organelle stains (DAPI [1 μg/ml; Abcam, Cambridge, UK]), MitoTracker (100 nM; Fisher) or FM4–64 (5 μg /ml; Fisher) according to the manufacturer's protocol, before (MitoTracker) or after (DAPI, FM4–64) treatment and DPBA staining.

Treated and stained cells on coverslips were then fixed with 2% v/v paraformaldehyde (in phosphate-buffered saline [PBS] pH 7) for 20 min, and the excess rinsed off using PBS before a further filter-paper blot to dry. One drop of 1% v/v n-propyl gallate mounting medium (in glycerol:PBS 60:40% v/v; ~5–10 μl) was added to microscope slides and coverslips mounted to slides. Coverslips were sealed to slides using nail polish, samples viewed by epifluorescence or confocal microscopy (LSM 880, Zeiss, Cambridge, UK) and images processed using ZEN software (Zeiss). Fixed samples were stored at -20°C.

All experiments were performed a minimum of three times, with a minimum of three biological replicates per experiment.

RESULTS & DISCUSSION

DPBA-enhanced epifluorescence microscopy

To determine whether DPBA-enhanced fluorescence emission could be successfully employed in *Dictyostelium*, cells were first viewed with and without DPBA treatment, via epifluorescence using the UV2A cube (Nikon) that limits excitation wavelength to 330–380 nm and emission to ≥420 nm, to check for autofluorescence. Cells grown axenically had been transferred to low-fluorescence medium and, following incubation, images were acquired using epifluorescence microscopy. Since there was no background cell autofluorescence (Figure 1A) in untreated wild type (AX2) cells nor cells treated with 0.1% (w/v) DPBA (Figure 1B), our protocol for visualizing plant native flavonoids [9] was modified for use with this model microbe. Three test flavonoids were chosen according to well-documented activity (as summarized above) in tissue culture, or from study using *Dictyostelium*, and concentrations for treatment were guided by those reports and parallel studies here of viability and life-cycle progression in *Dictyostelium* (Supplementary Figure 1). Cells were therefore treated with 50 μM kaempferol, naringenin and quercetin for 30 min. With addition of DPBA to prepared samples, epifluorescence could be used to view cells adhered to microscope slides that had undergone a time-course of treatment up to 60 min incubation with test flavonoid: it was possible to visualize kaempferol and quercetin after treatments of ~20 min (Figure 1C & D), and low fluorescence from naringenin was observed after ~40 min. Without the use of the flavonoid fluorescence enhancer, no signal was observed from *D. discoideum* cells treated with any flavonoid (Supplementary Figure 2) and no fluorescence was detected in solvent (DMSO)/DPBA controls (Supplementary Figure 2). The fluorescent probe therefore did permit *in vivo* visualization of flavonoid fluorescence for three flavonoids of therapeutic interest in this model microbe.

Dosage assay

Biological and transcriptional responses, such as cell rounding and toxin transporter upregulation were observed when *Dictyostelium* was treated with kaempferol, naringenin or quercetin at 10–50 μM, viability only declining for one compound, namely kaempferol with treatment of 100 μM (Supplementary Figure 1). To discern concentration-dependent effects by imaging, cells were therefore incubated (20 min) with 0–100 μM treatment and 0.1% DPBA: the resultant fluorescence signal in amoeba was enhanced in line with increasing concentration. As before, kaempferol (Figure 2) and quercetin were most easily detectable, at concentrations ≥25 μM, whereas naringenin fluorescence was not observed below 50 μM and was most visible at 100 μM (Supplementary Figure 3).

Time-course assay

The physiologically relevant 50 μM concentration was then used in a time-course experiment. Differential accumulation of flavonoids (Figures 1 & 2) again occurred in *Dictyostelium* as seen in other organisms and cell types [7,36], since quercetin fluorescence was visible after 10-min incubation, whereas for kaempferol visualization a treatment of at least 20 min was required. Naringenin required the longest incubation for detectable fluorescence, becoming most easily visible after 60 min of treatment (Supplementary Figure 4).

Confocal microscopy of co-localization dyes in conjunction with fluorescence enhancement

An increase in fluorescence could be the result of flavonoids bound to the surface of *Dictyostelium* cells rather than differential uptake. Therefore more sensitive, confocal, microscopy was employed and cell extracts were subjected to LCMS quantification to monitor flavonoid levels within cells. As noted above, dependent on cell type tested, flavonoids have been reported to localize to the nucleus or mitochondria, or the plant cell vacuole. To test if co-localization dyes could be used in conjunction with DPBA to allow subcellular location of flavonoids to be identified, *Dictyostelium* cells were treated with 50 μM of naringenin, quercetin or kaempferol, viewed using 488 nm excitation and 520 nm emission, along with the DAPI nuclear marker (excitation 405 nm, emission 461 nm), FM4–64 (excitation 561 nm, emission 737 nm) for plasma or vacuole membranes, or MitoTracker Red (excitation 561 nm, emission 599 nm) to stain the mitochondria [39–41].

Using the confocal microscope improved image quality when viewing DPBA-enhanced fluorescence and it was possible, as hoped, to visualize subcellular fluorescence from standard organelle markers as well as from the DPBA–flavonoid. A previous report on subcellular localization stated that a ▶

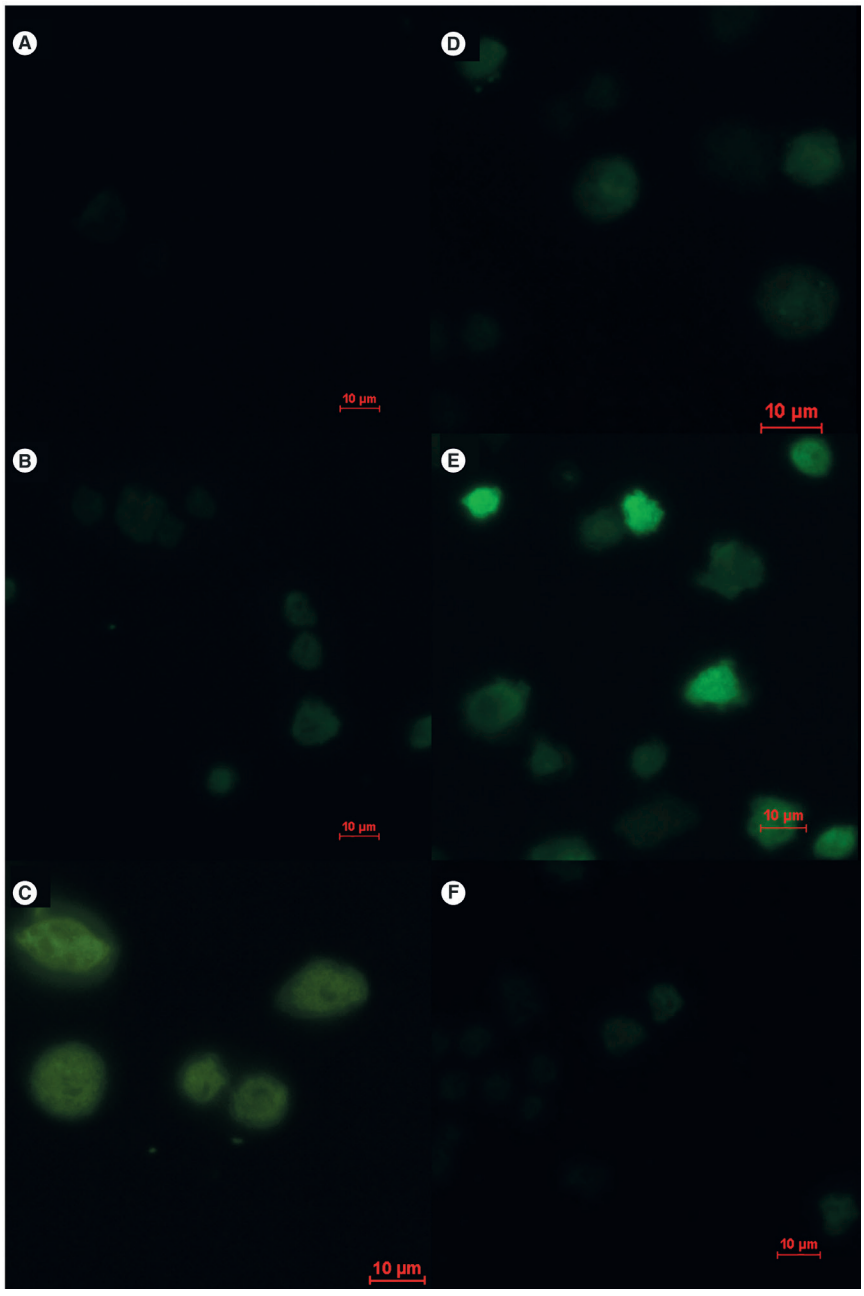

Figure 2. Epifluorescence microscopy of flavonoid-DPBA-treated *Dictyostelium discoideum* at concentrations of (A) 25 µM, (B) 50 µM and (C) 100 µM kaempferol; (D) 50 µM and (E) 100 µM quercetin; (F) 100 µM naringenin. For all treatments, see supplementary data. Images acquired using Nikon Eclipse 90i, Ex 470nm – Em 525 x60 lens.

▶ range of flavonols might reach the nuclei of cancer cells and induce DNA cleavage [42] and that quercetin reduced oxidative DNA damage and quenched free radicals in isolated DNA *in vitro* [43]. This co-treatment protocol, however, revealed that points of brightest fluorescence from the three test flavonoids did not occur at the nucleus, the fluorescence from kaempferol (Figure 3), naringenin and quercetin (Supplementary Figure 5) being largely independent of the DAPI emission in merged images. Here, as previously reported, MitoTracker Red (the import of which is dependent upon the organelle's membrane potential) stained *Dictyostelium* variably, also accumulating in circular 'submitochondrial bodies' in the cytoplasm [44,45]. However, MitoTracker–flavonoid colocalization was visible (Figure 3; see also Supplementary Figure 5), in agreement with the purported anti-oxidant properties of flavonoids: both quercetin and kaempferol were previously suggested to be cytotoxic to cancer cell lines by stimulating the mitochondria to overproduce ATP [46–48]. FM4–64 stained the plasma membrane and was internalized over time so that at 15 min it could be seen within *Dictyostelium* cells, at the contractile vacuole. The merged kaempferol and FM4–64 staining overlapped (Figure 3), in agreement with high kaempferol levels (Supplementary Figure 6) confirmed by LCMS in cell extracts (Supplementary Table 1).**Figure 3. Confocal microscopy of flavonoid-DPBA-treated *Dictyostelium discoideum* with concurrent use of cell markers. FM4-64 plasma membrane/contractile vacuole co-staining (15 min) after treatment with flavonoid (30 min). (A) Control, no treatment with flavonoid; (B) L–R: FM4-64, 50 µM kaempferol, merge; (C) L–R: FM4-64, 50 µM quercetin, merge. DAPI nuclear co-staining (followed 30 min flavonoid treatment); (D) L–R: 50 µM kaempferol, DAPI, merge. MitoTracker mitochondrial co-staining (for 30 min before 30 min flavonoid treatment); (E) L–R: MitoTracker, 50 µM quercetin, merge. See also supplementary data; images acquired with Zeiss confocal LSM 880 x63 lens. L: Left; R: Right.**

Parallel LCMS detection of flavonoids (Supplementary Table 1) showed naringenin was present in cell washes not cell extracts, and suggested significant quantities were not actively imported into *Dictyostelium* cells and that flavonoid fluorescence was imaged at the cell exterior. This was in agreement with the low fluorescence emission from naringenin seen in the dosage study (Figure 2), and the peripheral location of its reported target protein, PKD2, at the plasma membrane of both MDCK [49] and *D. discoideum* cells [50].

Use of fluorescence enhancement with specific flavonoid subtypes

Whereas three flavonoids were initially successfully imaged in *Dictyostelium*, *in vitro* assays of human cells detected DPBA-enhanced fluorescence from apigenin (in macrophages) [41] and hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone; in Caco2 cells) [7]. By contrast, our LCMS measurements showed that *Dictyostelium* cells were able to import the flavones apigenin and luteolin (3′,4′,5,7-tetrahydroxyflavone) and the flavanone hesperetin (the

latter being slow to accumulate, however), none of these were visible using confocal or epifluorescence microscopy of flavonoid–DPBA-treated *Dictyostelium* cells. The mechanisms for flavonoid influx into human cells have not been fully elucidated and observations have been reported to differ based on flavonoid, cell type and sample preparation, and this might equally apply to amoebae. There may be more passive diffusion of the less-polar aglycones [41] than of the more commonly found glycoside forms [51]. The extent of this is not known versus active transport and efflux, although our chemical inhibition studies suggest that flavonoid efflux is an active process (manuscript in preparation). The differences in flavonoid influx previously reported are in agreement with the treatment length and concentration here both being seen to influence flavonoid detection via DPBA-enhanced imaging. In this study, quercetin was rapidly transported by *Dictyostelium* cells, being visible at 25 μM after 10 min, and kaempferol was also detectable after 25 μM treatment for 20 min incubation (Figure 2). Naringenin was only minimally imported or not at all, requiring higher concentrations of 50–100 μM and requiring longer incubation for fluorescence to become visible (Figure 2, Supplementary Figure 2 & 3). Therapeutic use of naringenin is still possible, however, since it can exert a biological effect via the PKD2 target that is located at the cell boundary [49].

Chemical basis for DPBA conjugation

Searches of over half a century of literature yielded little detail of the mechanisms of DPBA–polyphenolic conjugation, but it was reported that the chemical structure of a compound determined both whether DPBA-enhancement of fluorescence emission occurs and its wavelength [30]. A hydroxyl group at the C3 position of the flavonoid skeleton (Figure 1) was suggested to be essential for fluorescence [39]. The flavanone naringenin is interesting in this respect, as it demonstrated weak DPBA-enhanced fluorescence in *Dictyostelium*, despite missing the hydroxyl group in the 3′ position of the C-ring (Figure 1), in contrast with hesperitin, which was not visible. Another report suggested a minimum of two hydroxyl groups were required [29] but, again, the chemical structure of naringenin fits this criterion for conjugation with DPBA. The low fluorescence of this compound *in vivo* may correlate with low concentrations bound to the cell surface, along with little import of naringenin.

Such differential accumulation of flavonoids is in agreement with other research, and underlines both the need for further studies of transport and bioavailability and the usefulness of *in vivo* imaging. Mouse liver hepatoma cells (HEPA-1c1c7), human umbilical vein endothelial cells (HUVEC), mouse neuroblastoma cells (Neuro 2A) and human epithelial colorectal Caco-2 cells [24–26] all showed different levels of accumulation of specific flavonoids, clearly pertinent to medical application of flavonoids as therapeutic compounds. The *in vivo* imaging method developed here is superior to LCMS and HPLC in not requiring cell fractionation nor solvent extraction of cellular components and compounds, and takes much less time. The evolutionary conservation of flavonoid targets in *Dictyostelium* and human cells and its popularity as a model organism means that this DPBA-enhanced *in vivo* imaging can be a useful adjunct to the necessary research on the mechanism of entry, selectivity and exit of flavonoids. In summary, DPBA-enhanced imaging can be used to reveal an association of flavonoids with *Dictyostelium* cells that can be viewed very simply with epifluorescence microscopy. For more detailed localization and dynamics of uptake, DPBA-enhanced confocal microscopy, and analytical chemistry for quantification, should be carried out. Identifying a compound's location in this model organism will help inform work on cellular targets, and ultimately progress the development of flavonoids from widely reported nutraceuticals to licensed therapies.

FUTURE PERSPECTIVE

Fluorescence imaging is a rapidly developing area. This most vital tool in science was established with longstanding techniques such as the use of ethidium bromide's intercalation into DNA to visualize nucleic acids in agarose gel electrophoresis; the binding of DAPI into the minor groove of DNA providing a subcellular (nucleus) marker; fluorogenic indicators permitting quantification of enzyme biochemistry; and native fluorescent compounds that indicate response to the environment – for example the ratio of chlorophyll a:b in plants. Confocal fluorescence microscopy has since become invaluable with the development of fluorescent reporter proteins, protein-interaction techniques such as BiFC, and further subcellular localization markers. Now, fluorescence utilizing nanoparticles and the range of fluorophores entering the market herald an era where all novel chemical entities and dynamic processes in cells can be viewed *in vivo* and in real time – for pharmaceutical science, this can illuminate, as in this study, the transport into and efflux from cells, which is often a key challenge in drug development.

Flavonoids have serendipitous attributes for imaging: the ability to enhance their fluorescence with the compound DPBA has been enormously useful for visualizing *in vivo* where these compounds are synthesized and transported within the plant. With the aim of reducing the need for studies using animals, DPBA protocols ▶

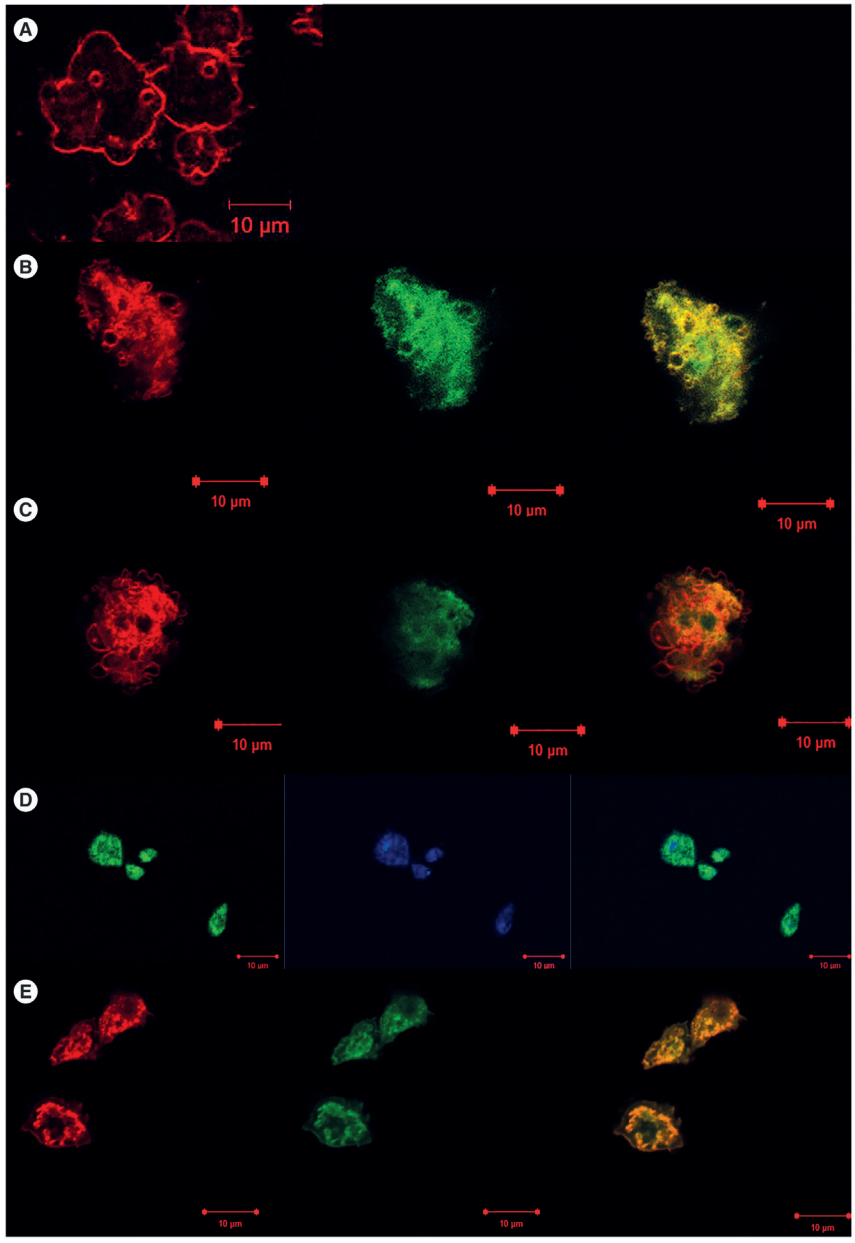

Figure 3. Confocal microscopy of flavonoid-DPBA-treated *Dictyostelium discoideum* with concurrent use of cell markers. FM4-64 plasma membrane/contractile vacuole co-staining (15 min) after treatment with flavonoid (30 min). **(A)** Control, no treatment with flavonoid; **(B)** L–R: FM4-64, 50 µM kaempferol, merge; **(C)** L–R: FM4-64, 50 µM quercetin, merge. DAPI nuclear co-staining (followed 30 min flavonoid treatment); **(D)** L–R: 50 µM kaempferol, DAPI, merge. MitoTracker mitochondrial co-staining (for 30 min before 30 min flavonoid treatment); **(E)** L–R: MitoTracker, 50 µM quercetin, merge. See also supplementary data; images acquired with Zeiss confocal LSM 880 x63 lens. L: Left; R: Right.

▶ were developed here for application in a model microbial eukaryote. This provides a tool for both flavonoid therapeutic development and for use of *D. discoideum* in preclinical studies. To exploit DPBA further, future work will delimit its activity *in vivo* with a broader range of polyphenolic compounds of therapeutic interest – of which there are many – and with their glycoside derivatives, which are more abundant in our diet. This fluorescent visualization of flavonoids that is being researched for medical application will permit high-throughput flow-cytometry and microplate assays, with the caveat that transport and destination of compounds requires detailed observation in parallel.

AUTHOR CONTRIBUTIONS

BTF carried out experimental work and helped write the manuscript; EPT planned experiments and wrote the manuscript.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The Rosetrees Trust part-funded this work. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0084

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- **Method; cell type-dependent localization; no uptake of flavonoid glycosides was detected.**

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