1) Method summary (1-3 sentences)

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 localisation to be monitored *in vivo*, using epifluorescence and confocal microscopy.

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.

2) 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 be synthesised 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

hypothesised 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 polymerisation [7] (Figure 1). For example, such compound specificity was described previously for the activities of plant polyphenols: only a subset of flavonoids affect 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]. 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): its action via the PKD2 protein, indeed, 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 signalling in cancer cells [17,18].

These polyphenolic compounds can be quantified in tissue extracts by UV–visible spectroscopy, high-performance liquid chromatography, mass spectrometry methods and nuclear magnetic resonance [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 visualising 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 visualised in mitochondria of monocytic leukaemia (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].

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 by 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 hypothesised 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 hypothesised 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 signalling 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 localisation of flavonoid-treatment and target *in vivo*. We found that differential uptake and targeting of therapeutically relevant flavonoids in the model amoeba could be visualised, and flavonoid localisation could be imaged along with other cell markers, using the DPBA probe.

Materials and methods

Dictyostelium 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 x 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 x 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 deionised 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 by rinsing, by dipping coverslips in 0.1M 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 0.1M 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 pH 7 (PBS)] 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; approx. 5–10 µl) was added to microscope slides and coverslips mounted to slides. Coverslips were sealed to slides using nail polish, and 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 experiments.

Results and 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 visualising plant native flavonoids (Thompson et al., 2010) was modified for use with this model microbe. Three test flavonoids were chosen according to well documented activity (as summarised 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* (Suppl. Fig. 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 visualise kaempferol and quercetin after treatments of approx. 20 min (Figure 1C,D), and low fluorescence from naringenin was observed after approx. 40 min. Without the use of the flavonoid fluorescence enhancer, no signal was observed from *D. discoideum* cells treated with any flavonoid (Suppl. Fig. 2) and no fluorescence was detected in solvent (DMSO)/DPBA controls (Suppl. Fig. 2). The fluorescent probe therefore did permit *in vivo* visualisation 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 (Suppl. Fig. 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 amoebae 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 (Suppl. Fig. 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 visualisation a treatment of at least 20 min was required. Naringenin required the longest incubation for detectable fluorescence, becoming most easily visible after 60 min treatment (Suppl. Fig. 4).

Confocal microscopy of co-localisation 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 localise to the nucleus or mitochondria, or the plant-cell vacuole. To test if colocalisation dyes could be used in conjunction with DPBA to allow subcellular location of flavonoids to be identified, *Dictyostelium* cells were treated with 50 µM 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 visualise subcellular fluorescence from standard organelle markers as well as from the DPBA–flavonoid. A previous report on subcellular localisation stated that a 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 (Suppl. Fig. 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]. MitoTracker–flavonoid colocalisation was visible, however (Figure 3; see also Suppl. Fig. 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 internalised 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 (Suppl. Fig 6) confirmed by LCMS in cell extracts (Suppl. Table 1).

Parallel LCMS detection of flavonoids (Suppl. 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]. Whereas 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, Suppl. Fig. 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 which can be viewed very simply with epifluorescence microscopy. For more detailed localisation 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.

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Protocol

Reagents

HL-5 Medium (ForMedium)
Lo-Flo Medium (ForMedium)
Microscope slides (VWR)
25mm coverslips (VWR)
Filter discs (Fisher Scientific)
Dimethyl sulphoxide (Sigma)
1x KK2 buffer
Flavonoids (Fisher Scientific; Sigma)
DPBA (Acros Organics)
0.1 M potassium phosphate buffer (PPB) pH 6.8
Ethanol (100%)
10x PBS pH 7.4 (25.6 g Na2HPO4·7H2O, 80 g NaCl, 2 g KCl, 2 g KH2PO4)
1x phosphate buffered saline (PBS) pH 7.4
5x PBS pH 7.4
2% v/v paraformaldehyde (Sigma)
1% v/v N-propyl gallate mounting medium (Sigma)
Nikon Eclipse 90i overhead microscope
Confocal microscope LSM880 (Zeiss)
MitoTracker (Fisher)
DAPI (Abcam)
FM4-64 (Fisher)

Preparation of cells

1. Grow cells axenically in 30 ml HL-5 medium at 22 °C 180 rpm for 2-3 days until a density of 10^7 cells/ml.
2. Retrieve 30 ml culture and proceed with KK2 wash to remove HL-5 medium:
a. Transfer culture to 50 ml falcon tube and pellet at 500g 4 min.
b. Discard supernatant, top up to 15 ml with 1x KK2 buffer, resuspend pellet, spin at 500g 4 min.
c. Discard supernatant, top up to 15 ml with 1x KK2 buffer, resuspend pellet, spin at 500g 4 min.
3. After the second KK2 wash, resuspend the cell pellet in 20 ml 1x KK2 and count cells. Once counted, resuspend cells to a final cell density of $5x10^5$ cells/ml in 30 ml Lo Flo medium in a sterile 250 ml conical flask.
4. Return cells to incubator for 24 h at 22 °C 180 rpm.
5. After 24 h, transfer cells to a petri dish containing sterile cover slips and 15 ml of Lo Flo, to a final density of $5x10^6$ cells/ml. Allow cells to attach to coverslips for 30 min.

Flavonoid treatment

1. Retrieve Petri dish containing cells attached to coverslips in LoFlo medium.
2. Proceed with your flavonoid treatment. Coverslips with attached cells were transferred to a fresh petri dish containing 15 ml Lo Flo medium + 50 µM of flavonoid. Petri dishes were returned to the incubator for 30 m at 22 °C.
3. After the 30 m incubation, retrieve coverslips and remove from petri dish. Rinse coverslips with 0.1M PPB pH 6.8 and blot dry with filter discs by capillary.

*a. *Hint: to rinse coverslips, dip slips into a beaker containing buffer.*

Excitation of tissues stained with 0.1% (w/v) DPBA in 0.1 M potassium phosphate buffer pH 6.8, 1% NaCl (w/v) (Hutzler et al., 1998) [53]

Staining with DPBA

1. Apply 0.1% DPBA staining solution onto coverslip, allow to stain for 5 m.
2. Rinse off DPBA stain from coverslips with 0.1M PPB pH 6.8 and blot dry with filter discs by capillary.

*a. *Hint: to rinse coverslips, dip slips into a beaker containing buffer.*

3. Fix coverslips with 2 % PFA for 20 m. Rinse off excess PFA solution using 1X PBS blot dry with filter discs by capillary.

*a. *Hint: to rinse coverslips, dip slips into a beaker containing buffer.*

4. To a clean microscope slide, place 1 drop of 1% n-propyl gallate mounting medium (approx. 5 – 10 µL) and mount coverslip to the slide. Seal coverslip to slide.
5. Image slide or store -20 °C until required.

Use of co-localisation markers

1.Follow supplier's guidelines for use of cell markers for live or fixed sample imaging.

Recipes

1X KK2 buffer (Eichinger and Rivero, 2006) [52]

1. 10X solution: Dissolve 22 g KH_2PO_4 (monobasic) and 7 g K_2HPO_4 (dibasic) in 1 L deionized water, autoclave.
2. Dilute 1:10 with deionized water for 1X concentration.

0.1M POTASSIUM PHOSPHATE BUFFER (PPB) pH 6.8

1. Combine 49.7 ml of 1M K_2HPO_4 and 50.3 ml of 1M KH_2PO_4 to achieve pH 6.8 at room temperature.
2. Make up to 1 L with deionised water.

1X PBS

1. 10X solution: to 800 ml of deionised water add 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2PO_4. Adjust pH to 7.4, top up to 1 L. Autoclave.
2. Dilute 1:2 for 5X, and 1:10 for 1X concentration.

0.1% DPBA STAIN

1. Dissolve 125 mg in 10 ml of 100 % ethanol to create 2.5 % stock
2. Dilute 2.5 % stock down to 0.1 % working solution using PPB pH 6.8

0.1% N-PROPYL GALLATE MOUNTING MEDIUM

1. Add 4 ml glycerol to 6 ml 1X PBS.
2. Dissolve 0.1 g n-propyl gallate in 10 ml solution prepared in step 1.

*a. *Hint: microwave for 5 s if experiencing difficulty with dissolving.*

3. Aliquot 1 ml and store in the dark at 4 °C.

<u>2% PARAFORMALDEHYDE</u>

1. Add 45ml 5X PBS to a beaker on hot stir plate at 50 °C
2. Add 2 g of paraformaldehyde to heated PBS.
3. Slowly, add 5M NaOH to dissolve PFA.
4. Cool solution on ice.
5. Top up to 80 ml with 5X PBS, adjust pH to 7.4, top up to 100 ml.
6. Aliquot 10 ml and store -20 °C.

Executive summary

DPBA-enhanced epifluorescence microscopy

- Addition of Naturstoff reagent A (DPBA) to *Dictyostelium* cells treated with flavonoids allows visualisation of flavonoid fluorescence *in vivo* by epifluorescence
- There is no autofluorescence from *Dictyostelium* cells grown in LoFlo medium, or treated with solvent or staining buffers

Visualisation of flavonoid uptake: dosage and time-course

- Flavonoid fluorescence can be enhanced so that treatment at biomedically relevant dosages can be visualised *in vivo*
- DPBA imaging is sensitive to flavonoid treatment concentration and differences in compound-specific uptake by *Dictyostelium* cells

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

- DPBA-enhanced fluorescence imaging by confocal microscopy is compatible with use of localisation markers (for nucleus, mitochondrion, plasma membrane and vacuole) in *Dictyostelium* cells
- Co-staining with organelle markers and DPBA allows imaging of subcellular destination of specific flavonoids

Use of fluorescence enhancement with specific flavonoid subtypes: chemical basis for DPBA conjugation

- Fluorescence enhancement is dependent on structural or environmental factors: DPBA variably enhanced imaging of flavanones, flavonols and flavanone. Necessary structural components and conditions for DPBA conjugation *in vivo* require further elucidation.