Reactive dyes for living cells — applications, artefacts, and some comparisons with textile dyeing.

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Abstract

An inclusive chemical definition of "reactive" dyeing of textiles is introduced, encompassing the CI Azoic, CI Mordant, CI Reactive, CI Sulphur and CI Vat dye application classes. Such reactive dyeing increases fibre retention of dye and makes application practically possible. The analogous application of dyes and fluorescent probes as microscopic stains in biology and medicine is outlined, focussing on using reactive fluorescent probes with living cells.

Parallels with textile dyeing are noted, e.g., enhanced probe retention and facilitation of probe application. However, the primary purpose of using reactive probes with live cells is detection of properties of biological systems: to identify biological structures and chemical/biochemical contents; assess biological functions and physicochemical properties; and determine changes in locations of cells and cell components. Problems occurring with such probes are outlined, particularly the problematic character of many standard protocols, and localisation artefacts arising with reactive probes whose reactant and product species are physiochemically significantly different.

This latter problem is explored via a case study of possible reactant/product artefacts with probes for reactive oxygen species. Comparison of experimental observations of probe localisations with the localisations predicted using QSAR modelling indicates that such artefacts can occur with a significant proportion of chemically diverse, widely used, commercially available probes, as well as with experimental compounds reported in the literature. A graphical flowchart is provided to assess possible occurrence of reactant/product artefacts arising with reactive fluorescent probes localising in various organelles of living cells.

Keywords: artefact, fluorescent probe, reactive dye.

Contrasting perspectives on "reactive" dyeing of textiles

Considered in the most general chemical sense, reactive dyeing of textiles is a colouration process in which the colourant or colourant precursor (reactant) which is applied to a substrate does not have the same chemical structure as that of the colourant (product) retained in the substrate after dyeing is complete. In this sense acid dyeing of wool, basic dyeing of acrylic fibres, or the colouration of cotton by direct or disperse dyeing, are not reactive processes. So CI Acid, CI Basic, CI Direct and CI Disperse dyes are not reactive dyes. In keeping with this perspective the CI Reactive application class is restricted to dyes which form covalent bonds with groups on the textile substrate.

However, in terms of the above inclusive concept of reactive dyeing, CI Azoic, CI Mordant and CI Sulphur dyes are all reactive. Dyes of all these classes are applied as precursors, from which reaction products are generated within the fibre. CI Vat dyes are also reactive, however with the complication that the molecular species added to the dyebath is the same as the one retained in the fibre — whilst the alkali soluble~~, fibre substantive,~~ reduced leuco form is generated in the dyebath and is converted back to the original form within the fibre. Consequently, textile dyeing reactive dyes in the wider sense are not restricted to the CI Reactive application class. Figure 1 provides examples of these distinctions, comparing a CI Reactive dye with "reactive" textile dyes of two other application types.

Figure 1 near here

The function of reactivity in the case of the CI Reactive application class is simple: to provide an increase in retention of dye in the fibre, leading to the desired high fastness. The aim of generating a colouration of high fastness also applies to the reactive dyes as defined in the inclusive way. Thus the products of azoic dyeing are insoluble pigments trapped within the fibre, as are the products of vat dyeing and the oxidized, water insoluble sulphur dyes. The chromium complexes of mordant dyes are also less easily removed from the fibre than the parent acid dye. However, reactivity sometimes also serves a second function: to make the application of a colourant to the fibre practically possible. Thus, as illustrated in Figure 1b, the reduced form of a vat dye is ionic, and thus water soluble, unlike its oxidized product. A similar solubility difference is seen in azoic dyeing (Fig. 1c), in which the azoic coupling component and diazonium salt reactants are water soluble, unlike the resulting azo dye product; and also in sulphur dying, which involves applying a reduced, water soluble

derivative to the fibre, with subsequent oxidation giving rise to a coloured product insoluble in water. Accounts of all these processes are available in a recent monograph [1].

Dyes and fluorescent probes used as microscopic stains in biology and medicine

Dyes, fluorescent and otherwise, have long been used to assist microscopic investigation of biological specimens. Originally, such studies were mostly of *dead* materials, involving biopsies of cells, tissues and organisms; for a general historical account see [2]. The objectives of such colouration were and are — in contrast to much textile dyeing — always functional, not aesthetic.

A minimal objective is to visualise a specimen, since much light microscopy requires the use of optically thin materials, which are colourless or only very palely coloured. These specimens have typical thicknesses in the range 5–50 μm, and are usually generated from the original cells and tissues by smearing or sectioning. Most staining systems used in biology and medicine, however, also provide selective colouration of different biological entities. The resulting colour coding then provides information on the structure, composition and function of the specimen. This topic has been discussed previously in this journal (e.g., [3] [4]), and for an overview of how such staining procedures are currently applied in pathology see a recent handbook [5]. Some methods of this type do in fact make use of reactive dyes, for instance the Feulgen nucleal procedure for detecting DNA, and the periodic acid-Schiff procedure for demonstration of polysaccharides.

However, dyes are also applied to *living* cells and organisms. Indeed, over the past several decades the commercial availability of sophisticated fluorescence microscopes of various types has encouraged a flourishing of this approach. Consequently, a wide variety of fluorochromes are currently used to stain living cells, for a recent survey see [6]. Most of these agents are fluorescent dyes, which are termed fluorescent probes by biologists and biomedical researchers. Many of these dyes are non-reactive. Of currently used reactive probes none are members of the CI Reactive dye class, although such dyes have been investigated for biomedical applications in the past.

As with the earlier dyes, such probes are used to simultaneously answer two quite different questions. Namely, is a particular entity present; and if so, where does that entity occur within a cell or tissue? Occasionally additional questions are asked, for instance: how much, or how many, of the entity of concern is/are present? Or how does the entity change

with time? Table 1 gives an indication of the wide variety of information obtainable in this way. When inspecting this table note that some, but not all, probes are reactive.

Table 1 near here.

In the present account, only *reactive* dyes which are used as *microscopic* stains of *living cells and tissues* are considered. This article therefore largely ignores non-imaging dyes which do not localise within a biological substrate, such as using the tetrazolium salt → formazan transformation to assess cell viability without intracellular localisation of the coloured reaction product [7]. Also excluded is the use of dyes to obtain localisation/composition information on the macroscopic scale — e.g., of complete, large organisms, such as when assessing peroxynitrite in live zebrafish [8] or tracking the movement of free-living mosquitos [9]. Accounts of the wider uses of fluorescent probes are of course available, including one in this journal [10].

Why are some fluorescent probes reactive?

As noted above, reactivity of textile dyes serves not only to increase the fastness of dyes for fibres but also, in some cases, to assist their application to the fibres. Since, analogously to the case of textile dyes, some fluorescent probes are reactive and some not, we can enquire what is the function of reactivity in the case of biologically applied probes? There are in fact three quite distinct functions.

For some probes the function is the same as that noted for textile dyes: *to prevent loss of probe from the specimen following removal from the staining solution*. Consider the selective staining of mitochondria in the cytoplasm of living cells using the non-reactive dye Rhodamine 123. As a living cell is labile, such a preparation has a restricted lifetime, and transient staining outcomes must be recorded photographically. One alternative is to stabilise the cell structure by "fixation" (see [5] chap. 4). Unfortunately such protein-denaturing treatments kill the cell. The consequent elimination of the mitochondrial membrane potential, which drives the mitochondrial uptake of the Rhodamine 123 probe, permits the dye to diffuse away, destroying information concerning localisation. However, this problem can be avoided by use of a probe which forms covalent bonds with the proteins within the mitochondria. Then, when such proteins were insolubilized by fixation, the probe is retained *in situ*. The case of MitoTracker Green FM in Fig. 2a provides an example of this approach. Permanent attachment of a probe to some long-lived cell component is also

required when tracking changes in the position and movement of a cell during development of a multicellular organism; or when tracking changes in the location of an organelle within a living cell. Examples of reagents used for this purpose include CellTracker Blue CMAC, as shown in Fig. 2b. Another quite distinct mechanism for retaining a colourant within the cell arises when the probe is present within the target as a pigment, being insoluble in water and in any subsequent processing solvent. This strategy has also been adopted with fluorescent probes and live cells. For instance, the presence of the enzyme acid phosphatase can be demonstrated in the lysosomes of living cells by applying a water soluble naphthol ester and a water soluble diazonium salt to the cell, which results in the selective formation of an insoluble fluorescent azo pigment within lysosomes [11].

That such fluorescent probes are analogous to CI Reactive dyes and CI Azoic colourants, respectively, is clear. However, there are two additional pay-offs of reactivity in fluorescent probes with biomedical applications.

Figure 2 near here

The first of these, again paralleling some textile dyes, is *to facilitate entry of probes into the cells*. In biomedical contexts, however, this tactic is most commonly used when a probe is too hydrophilic to pass through the lipophilic cell membrane by passive diffusion, and so is membrane impermeant. A common response to this problem is to produce a more lipophilic derivative of the probe, which is membrane permeant. The derivative is selected such that, once inside the cell, some common cell metabolic process regenerates the "active" probe. Thus, carboxylic acids or acidic hydroxyl compounds are esterified to produce lipophilic methyl or acetoxymethyl (AM) esters. Within the cell a variety of esterases regenerate the original active compound, as shown in Figure 3a. This process has been applied for the introduction of probes detecting a variety of targets, see for instance Fluorescein diacetate and Rhod-2 AM in Table 1. A second modification used to increase membrane permeability is to convert the probe to a reduced form, as shown in Figure 3b. This has been used to introduce the cationic dye Ethidium into live cells in the form of uncharged Hydroethidine. Intracellular oxidation then converts this to the Ethidium species, which is retained in the cell nucleus because it intercalates into double stranded nucleic acids, as shown in Figure 3b. Note that esterifying a single carboxylic acid to its methyl ester can result in a lipophilicity increase equivalent to adding several methyl groups because it removes the full negative charge of the carboxylate, which is the main species at neutral pH; and reducing Ethidium to Hydroethidine causes a comparable increase in lipophilicity by removing a positive charge. A variant on this mechanism is the staining of lipid droplets in

live cells with Phenazine Methosulphate (PMS) — or more precisely with Methylphenazine, the reduced, lipophilic species generated from PMS in the plasma membrane by the action of NAD(P)H reductase [12]. In this case the reaction product is both more membrane permeable than the reactant and also has an affinity for the cellular target which the reactant does not.

Figure 3 near here

Such changes are functionally equivalent to certain textile dyeing tactics. Indeed the second and third examples, above, have parallels with vat dyeing. However, with fluorescent probes the modifications increase access to the cellular target by increasing membrane permeability, and indeed with PMS generate the species with affinity for a cell constituent; whilst in azoic and vat dyeing it is the formation of a soluble dye species which is important.

The final, and often the most important, advantage of using reactive fluorescent probes with live cells is however *to allow identification of intracellular chemical and biochemical species*. In this case the role of reactivity is to provide selective generation of an optical signal at the site of the target substance within the cell. The classes of target are numerous. To expand on Table 1 whilst not attempting an encyclopaedic listing, note that selective staining provided by such probes can identify a variety of targets including:

Enzymes — e.g., dehydrogenases, esterases, oxidases, phosphatases.

Metal ions — e.g., Ca^{2+}, K^{+}, Mg^{2+}, Na^{+}, Zn^{2+}.

Molecular fragments — e.g., $–NH_2$, –SH.

Nucleic acids — DNA, RNA.

Reactive oxygen species — e.g., hypochlorite, singlet oxygen, superoxide anion radical.

Small molecule signalling compounds — e.g., H_2S, NO, SO_2.

The range of chemical reaction mechanisms exploited to provide the basis of such reagents is wide. Even a single target may be identified by reactive probes utilizing a range of chemistries. To illustrate this, Figure 4 shows some of the reaction mechanisms exploited by reactive probes for hydrogen sulphide; and a summary of the various pay-offs of reactivity when applying textile dyes and fluorescent probes is provided Table 2.

Figure 4 near here.

Table 2 near here

Problems arising with reactive fluorescent probes applied to live cells

So far we have focussed on the advantages of reactive probes, since microscopy remains — 350 years after Leeuwenhoek, and 150 years after the 19th century flowering of synthetic dyestuffs and of the commercialisation of microscopes — a key investigatory technology in biology and medicine. However, all pennies have two sides, and we now turn to some problems arising with this approach.

Artefacts and other problems arising when using fluorescent probes with live cells have been discussed on many occasions (e.g., [13] [14] [15]). Rather than reprise such accounts, we here focus on two topics, of particular relevance for reactive probes, which to date have not been widely discussed. These topics are:

1. That the protocol-driven application of a probe — a routine response when wishing to answer the question "How do we check if entity X is present?" — is unlikely to provide a universally valid approach, and will sometimes actively mislead.
2. That for organelle-specific probes the potentially confusing consequences of marked physicochemical differences between the reactant and product dye species has rarely been discussed. As this could be a major problem specific to reactive probes, it will be treated in some detail, and illustrated with case examples.

Standard protocols are more problematic than commonly assumed

First note that since protocols are widely considered "mere technique" it is commonplace for key details of the staining protocol to be omitted from published work, even in the major journals. For instance, the nature of the solvent and of any co-solutes used when applying a probe to the live cells may not be specified. Nor is the temperature of application or any pretreatments of the cells necessarily given. Moreover, the solvent used for any post-staining washing, along with the timing and temperature of such a step or steps, are equally likely to be omitted. It will be apparent that such omissions significantly increase the difficulties of understanding the mechanisms by which selective staining of a cell target is achieved.

Two exemplar problems will now be discussed. The first is widely problematic, the second is especially relevant for reactive probes.

Enhancing the membrane permeability of hydrophilic dyes by applying the dyes in the form of AM or methyl esters is a widely used strategy, mentioned above. However, some AM esters of calcium probes, such as Fura Red, are so hydrophobic that they would be expected to be trapped in the plasma membrane, and so fail to enter the cell [16]. Nevertheless, with a suitable protocol they prove to be satisfactory reagents. Other probes, such as Fura-2, are membrane permeable but nevertheless their staining intensities are protocol dependent. A common factor in both these puzzles can be the presence or absence of serum albumin (SA) in the incubation medium. Significantly, SA has esterase properties. Consequently, when using an incubation medium containing SA, partial ester hydrolysis of Fura Red AM can occur in the extracellular incubation medium, generating a mildly lipophilic species which is membrane permeable [14]. On the other hand, whilst Fura-2 AM is membrane permeable, if the incubation medium contains SA then, if the solution is not used soon after preparation, the ester may already be partially extracellularly transformed into a hydrophilic, membrane impermeable, free acid [17]. A related problem is that esterase activity is temperature dependent, so if the temperature of incubation is not as in the protocol then the degree of ester hydrolysis, and hence membrane permeability, will be uncertain.

A second protocol problem arises when using a standard organelle stain to check, by the observation of co-localisation, that a novel reactive probe has indeed accumulated in the desired target organelle. This involves administering a standard probe, already known to localise in the appropriate site, together with the novel probe, to see if both accumulate ("co-localise") at the same part of the cell. However, it is usually the case that only the co-localisation of the standard probe with the reaction *product* of the probe in the target is checked, or can be checked. Unfortunately, such a control procedure does not exclude the possibility that the reaction has occurred at some site other than the target organelle, but that the product has then relocated to the target organelle. Probably due to the difficulties of assessing this, possible localisation artefacts of this type have received little attention to date. Since such relocation artefacts could arise with reactive probes, this is considered in more detail below.

Consequences of physicochemical differences between a reactant and its product

Cellular localisation of probes is substantially influenced by their physicochemical properties. Localisation within a specific organelle typically depends on the probe possessing a

particular combination of amphiphilicity, electric charge and hence pK_a, lipophilicity, and overall and aromatic system size. Consequently, probe localisation can be predicted using appropriate quantitative structure activity (QSAR) models [10]. Note that some probes mimic metabolites, and so their localisation is also influenced by transporters and selective pores in cell membranes, but such complications will not be discussed here.

To describe the possible localisation artefacts clearly, consider a reactive probe A, intended to detect entity X in organelle J. Further, consider the possibility that the physicochemical properties of the product species of the probe ($A_{product}$) are very different to those of the reactant species ($A_{reactant}$). Under these conditions, although the visible product of staining is seen in organelle J, generation of $A_{product}$ may have occurred in some other site, say organelle K, but $A_{product}$ has then relocated. Consequently, when using a routine staining protocol and routine control stain, staining of organelle J will be taken as evidence that entity X was present (correct) in organelle J (not necessarily correct). Accurate reporting of cell composition and localisation thus require both reactant and product species of a reactive probe to preferentially accumulate in the same organelle. This possible error is represented diagrammatically in Figure 5.

Figure 5 near here

A reactant/product localisation artefact which has been reported arises when using the MTT Tetrazolium reagent to assess cell viability [18]. This standard procedure is based on the administration of the cationic, colourless and membrane permeable MTT Tetrazolium salt to living cells. Cell localisation QSAR models indicate that this reactant species will accumulate in the endoplasmic reticulum (ER). At this site a more lipophilic, reduced and electrically neutral MTT Formazan product is generated enzymatically. This product then relocates to lipid droplets and to the lipid-rich Golgi apparatus, as shown in Fig. 6. Changes in key physicochemical properties driving this relocation are shown in Table 3.

Fig. 6 near here

Table 3 near here

As reactant/product localisation problems can in principle arise with routine commercially available probes as well as when investigating new compounds, such artefacts could constitute a significant problem with both the application and the development of reactive probes. For this reason, additional case examples are considered below. However,

in the interests of brevity, only probes reporting on reactive oxygen species (ROS), and localising in lysosomes or mitochondria, will be considered in any detail.

Case study of possible reactant/product artefacts arising with probes for ROS

Current interest in the detection of ROS in biological and medical fields is considerable, since ROS are associated both with various pathological conditions and also with signalling processes. As a consequence, preparation and application of small molecule probes for ROS has been reviewed on a number of occasions (e.g., [19] [20] [21]). Most widely applied probes for detection of ROS are derivatives of Fluorescein, rhodamine or rosamine that can be chemically reduced to non-fluorescent leuco compounds (e.g., [6] [22] [23]). Such dihydro derivatives are readily oxidized back to the parent dye by some ROS, and thus serve as fluorogenic probes for detecting oxidative activity in live cells. Such dihydro reactants are inevitably less hydrophilic than their oxidized reaction products. In addition, some of these probes are administered as lipophilic AM or methyl esters, and in these instances there is a chain of reactions leading to the final product, with two possible precursors and one final product. Multiple reactant/product localisation artefacts are therefore possible in such cases.

Note in passing that significant problems of other types also arise with ROS probes. For instance, as a ROS probe will compete with intracellular antioxidant for the ROS species, changes in fluorescence could reflect changes in competing antioxidants rather than changes in the amount of ROS. Moreover, probes for ROS do not all detect the same ROS, e.g. "intracellular oxidation of dihydrogen-calcein … detects a limited spectrum of ROS" [24]. As a final example consider that, due to photooxidation, several reduced ROS probes such as those deriving from Rhodamine 123 and Tetramethyl-rhodamine Methyl Ester can also act as ROS generators [25] [26].

One way of evaluating the possibility that a reactant/product pair will give rise to a localisation artefact is to make use of QSAR modelling to predict the localisation of both reactant and product species. This approach will now be described, starting with the specification of an integrated flowchart for predicting localisation artefacts for probes localising in lysosomes and mitochondria.

Predicting the occurrence of reactant/product localisation artefacts

Such predictions can be made using a two-step procedure. Step 1 involves use of a flowchart derived from published QSAR localisation models (see [10] for a summary account), which inputs structure parameters for a probe and outputs predictions of the cellular localisation of reactant and product species of fluorescent probes. Step 2 involves a second flowchart, which inputs the predictions of step 1, and outputs predictions of whether the observed localisation of a probe is likely to be reliable or is artefactual. This two-step procedure is illustrated in Fig. 7.

Figure 7 near here.

These procedures will now be carried out on some set of reactive probes whose observed localisations have been reported. Following this we will see if reactant/product localisation artefacts are actually expected, and, if so, we can obtain some estimate of their possible frequency.

Assessing occurrence and frequency of reactant/product localisation artefacts

For this to be carried out, an unbiased sample of reactive probes was required. Fortunately, such a listing had been assembled by one of us (HZ) for reasons entirely unrelated to the questions considered in this present article. This comprised publications describing small molecule fluorescent probes of many kinds, published in major, peer-reviewed journals. These compounds were based on a variety of fluorophores, and a variety of chemistries were involved in the reactant/product transformations. One criterion for including a probe in the list was provision of a micrograph illustrating its cell localisation. From this listing was obtained a set of papers describing 29 reactive probes for ROS species which were observed to localise in lysosomes or mitochondria. After investigating the 29 compounds using the procedure summarised in Fig. 7, it was concluded that:

a. Reactant/product localisation artefacts were predicted for more than half (16/29) of the probes studied.
b. These included both commercially available compounds and novel/experimental probes.
c. These probes were based on a variety of fluorophores.
d. Probes for the same target often involved different reaction chemistries.
e. Probes for both lysosomes and for mitochondria were listed.

Although the sample investigated were all probes for ROS, and the sample size is small, the implication that reactant/product localisation artefacts may be frequent is disturbing. Consequently, a less systematic inspection was carried out of 65 other compounds in the reactive probe list, localising in lysosomes and mitochondria. These had been used for the demonstration of other types of cell entities, including a variety of inorganic anions and metal cations, enzymes, and a number of small biomolecules including the signalling compounds H_2S and SO_2. This survey found that the occurrence of potential reactant/product localisation artefacts was widespread, and was not restricted to probes of ROS. The overall incidence of possible reactant/product localisation artefacts identified by this survey was around a fifth of probes listed.

As an illustration of this approach, consider the commercially available ROS probe H_2-Calcein AM, which was predicted by the flowchart to accumulate in the plasma membrane. So how did such probe behaviour result in the experimental observation of mitochondrial staining, as reported [27] in the literature? Perhaps because the plasma membranes of many cell lines contain oxidases ([28]). Oxidation of H_2-Calcein AM in the plasma membrane would give rise to various membrane permeant weak acids, some of which are predicted to accumulate in mitochondria.

Conclusions. Many types of textile dyes and fluorescent probes are reactive. Such probes — which are used to obtain information concerning the structure, contents, properties and activities of living cells — show many parallels with reactive dyes for textiles. However, the biological application of reactive fluorescent probes is not without problems, including the generally unacknowledged case of reactant/product localisation artefacts. Occurrence of such artefacts can however be predicted using a simplistic, but rapid, algorithmic trouble shooting guide.

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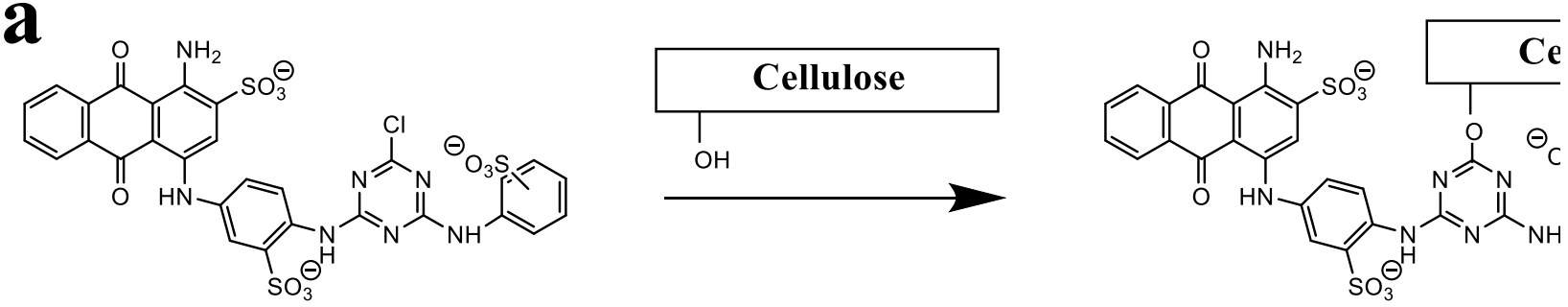
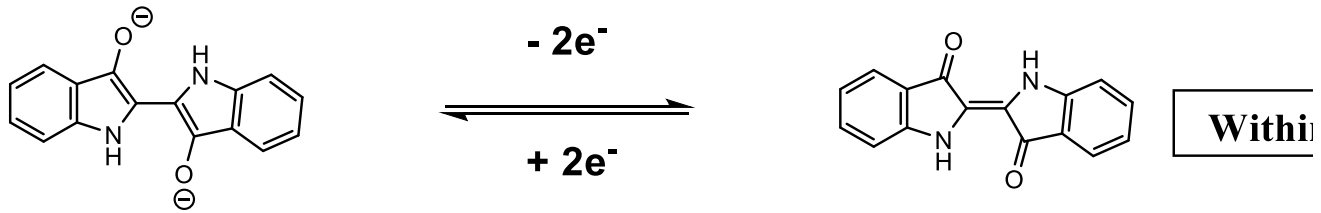
Figure 1. Examples of different types of "reactive" textile dyes. **a**. CI Reactive Blue 2, a dye of the CI Reactive application class, whose retention within cellulosic fibres is due to formation of covalent bonds between dye and textile polymer. **b**. Indigo (CI Vat Blue 1) which is taken into textile fibres as a water-soluble reduced species but, following oxidation, is retained as an uncharged and insoluble pigment. **c**. Formation of an insoluble azo dye within a textile fibre from the azo coupling agent Naphthol AS (CI 37505, CI Azoic coupling component 2) and the diazonium salt Fast Red GG (CI 37040, CI Azoic Diazo Component 9). For more details on such reactions see [1].

a



b



c

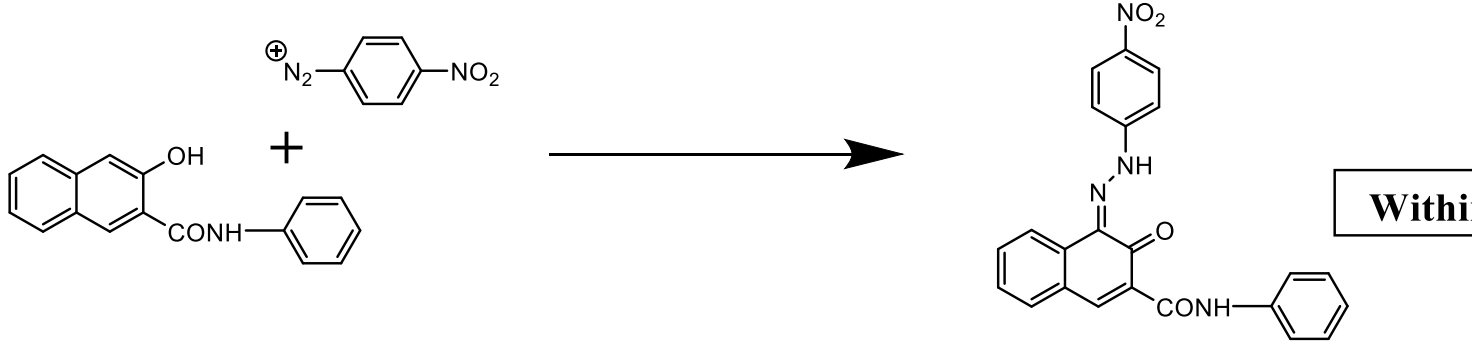
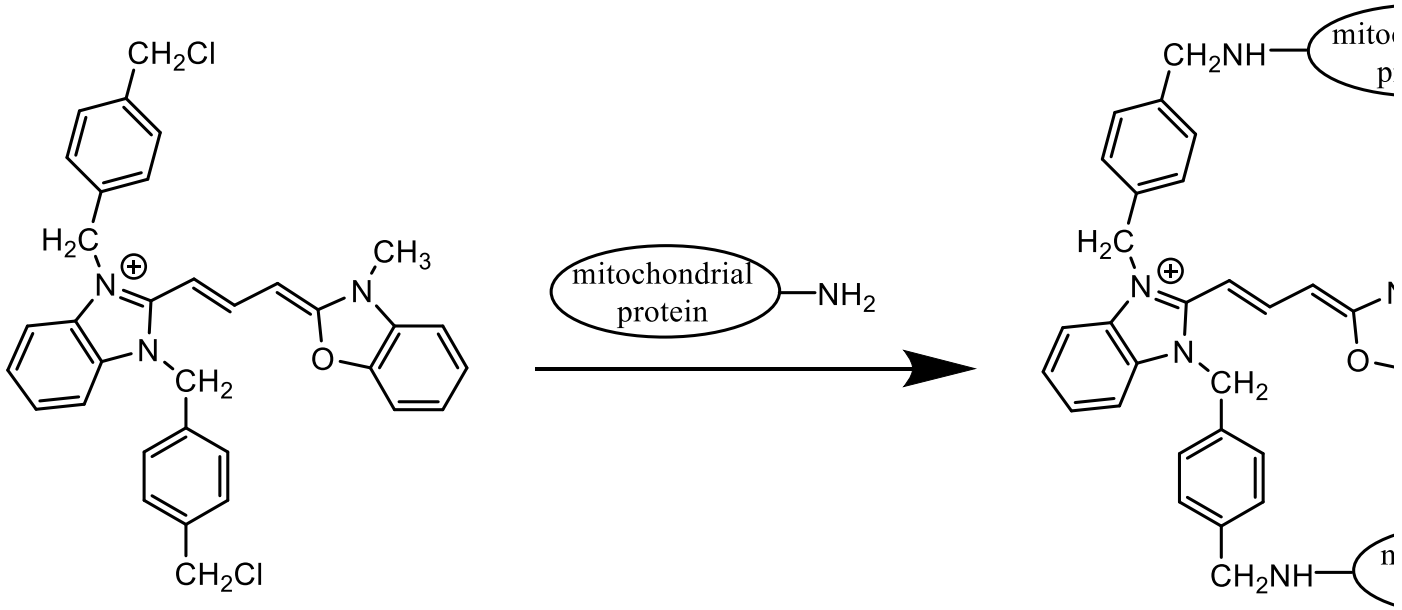


Figure 2. Examples of fluorescent probes in which reactivity serves to enhance retention within the target. **a**. MitoTracker Green FM, whose chloromethyl substituent forms a covalent bond with mitochondrial proteins following the probe's selective uptake into that organelle, which proteins plus attached probe are immobilised after fixation of the cell following staining. **b**. CellTracker Blue CMAC, whose chloromethyl group forms a covalent bond with cell proteins, providing a stable attachment permitting long-term cell tracking. For more details on these reactions see [6].

a


CH2Cl
H2C
N
CH3
N
N
CH2
O
CH2Cl
mitochondrial
protein
NH2
CH2NH
mito
p
H2C
N
N
CH2
O
CH2NH
n

b

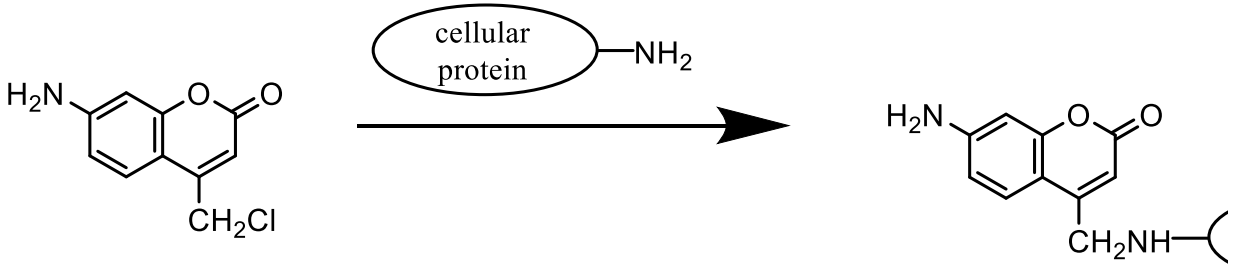
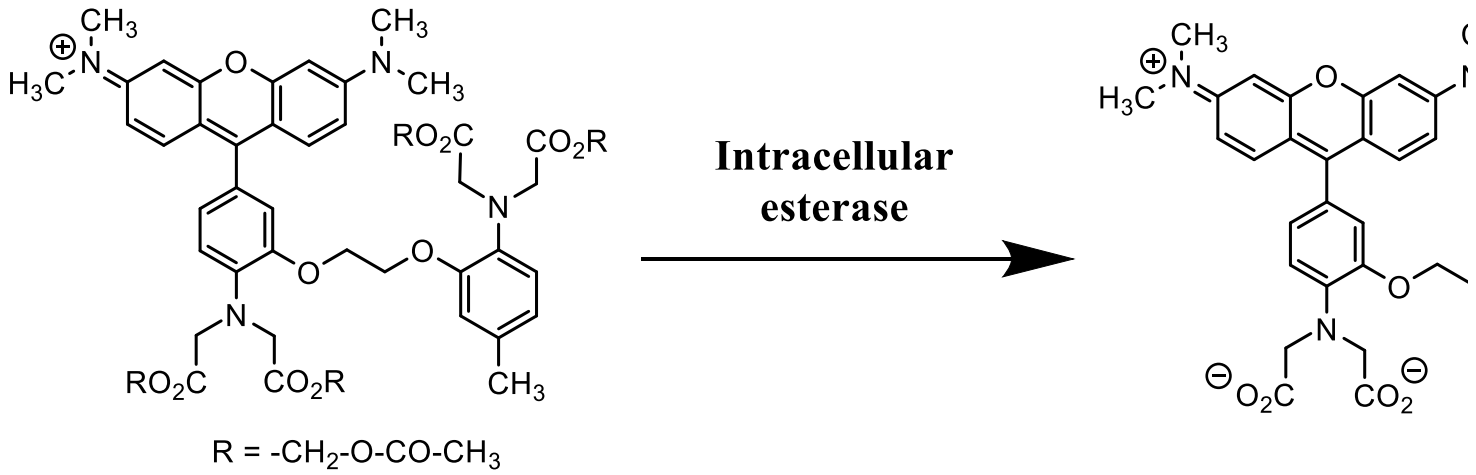

H2N
O
O
CH2Cl
cellular
protein
NH2
H2N
O
O
CH2NH

Figure 3. Examples of fluorescent probes applied in membrane permeant form which can enter cells, and there be converted by cell biochemical processes into "active" compounds. **a**. Conversion of lipophilic, membrane permeable Rhod-2 AM by cell esterase to the hydrophilic, calcium ion binding agent Rhod-2. **b**. Conversion of lipophilic, membrane permeable Hydroethidine by intracellular oxidation into the hydrophilic, DNA binding dye, Ethidium. For more details on these reactions see [6].

a


Intracellular
esterase
R = -CH2-O-CO-CH3

b

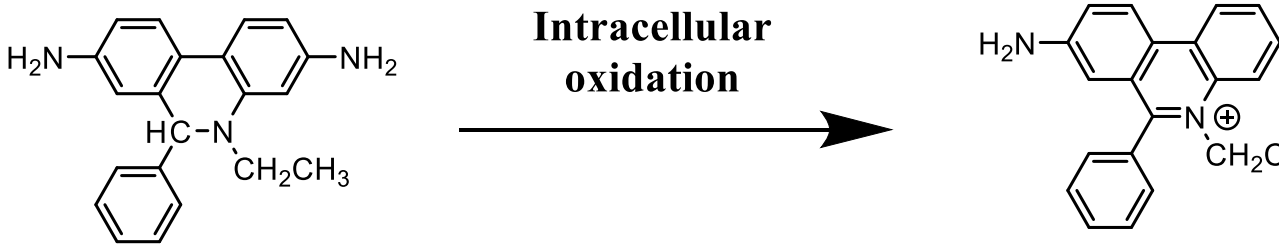
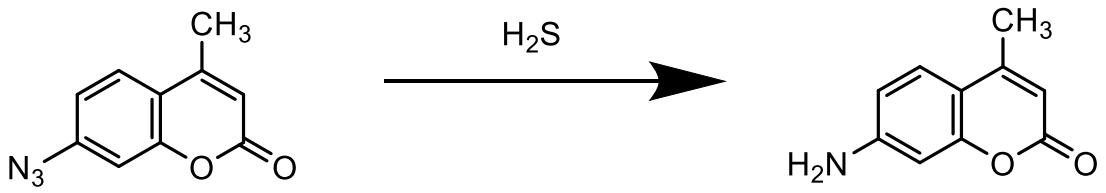
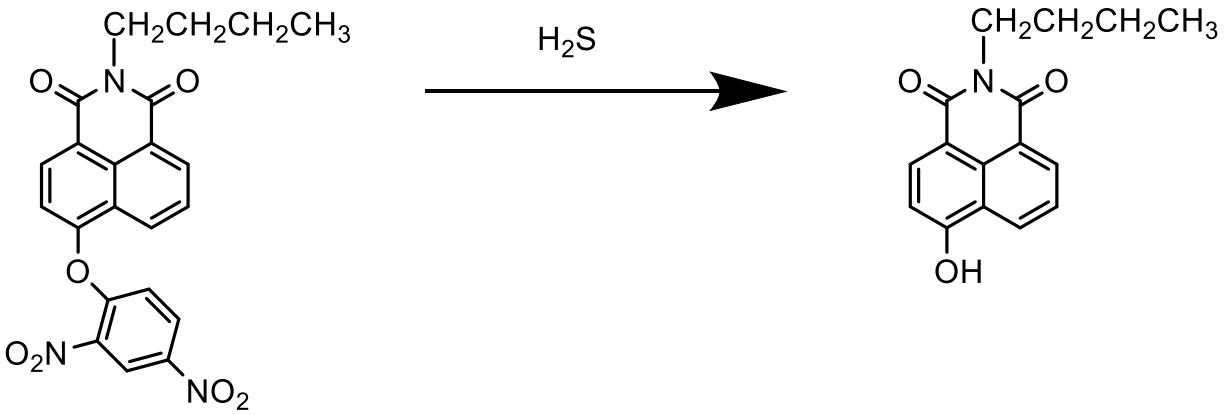

Intracellular
oxidation

Figure 4. An illustration of the diversity of chemistries used in reactive fluorescent probes, using examples of probes which image hydrogen sulphide in living cells. **a**. Chemoselective reduction of the aryl azido group of AzMC to yield a fluorescent amine. **b**. Thiolysis of the dinitrophenyl ether of NI-NHS yielding a fluorescent naphthalimide. **c**. Nucleophilic attack on the ester of HS-Cy, from which a fluorochrome is formed in the final reaction step. For more details on these reactions see a recent review [29].

a


CH3
H2S
N3
O
O
CH3
H2N
O
O

b


CH2CH2CH2CH3
O
N
O
O
H2S
O2N
NO2
CH2CH2CH2CH3
O
N
O
OH

c

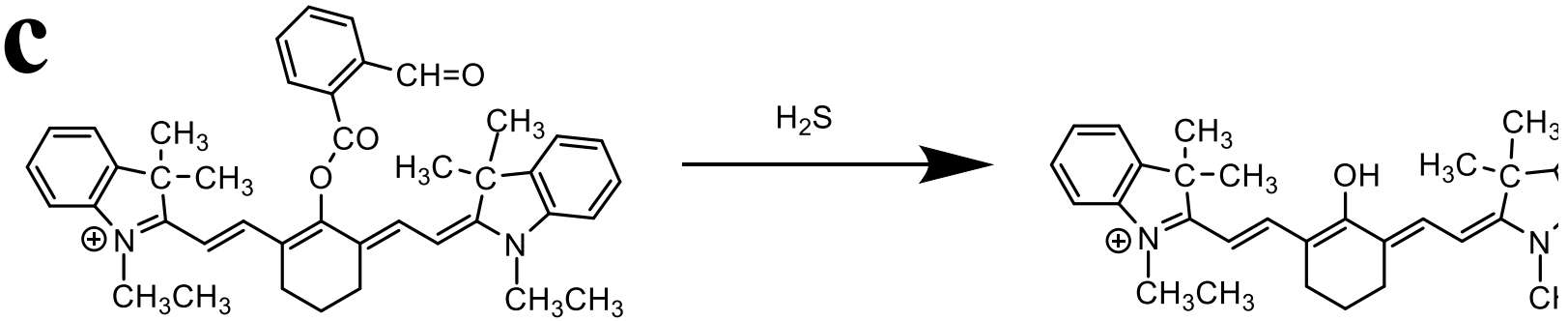

CH=O
CO
O
CH3
C
CH3
H3C
C
CH3
N
CH3CH3
N
CH3CH3
H2S
CH3
C
CH3
OH
H3C
C
CH3
N
CH3CH3
N

Fig 5. How reactant/product localisation artefacts can arise. The scheme shows two possible pathways of the reactive probe A, which is supposed to identify an entity X in organelle J. When $A_{reactant}$ and $A_{product}$ are physicochemically similar, they will both accumulate in the same organelle. Hence if X is present in organelle J staining will occur in organelle J. However if $A_{reactant}$ and $A_{product}$ are physicochemically very different they may localise in different organelles. Thus whilst $A_{reactant}$ may initially accumulate and react in organelle K, $A_{product}$ may finally relocate and accumulate in organelle J.

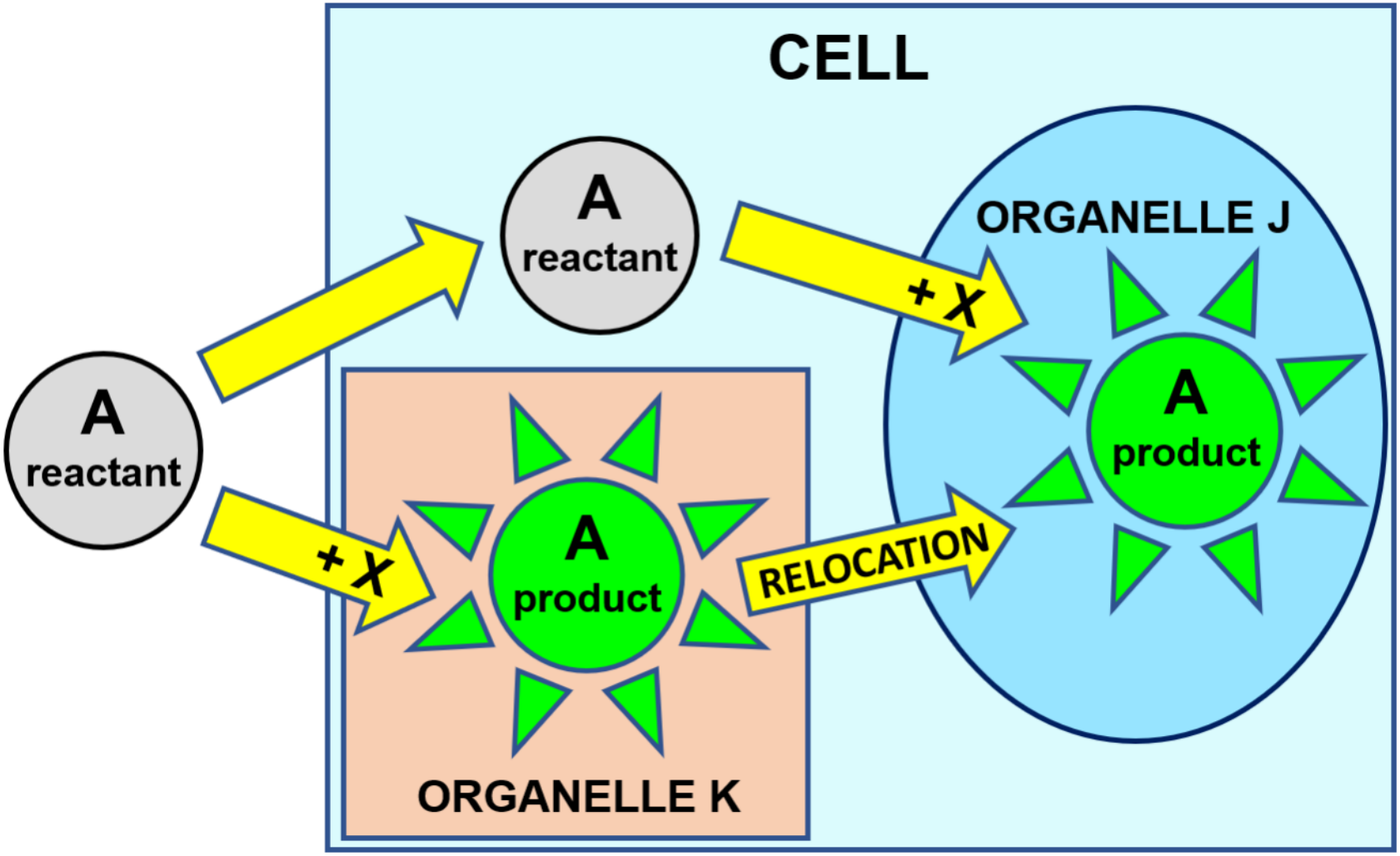

CELL
A
reactant
A
reactant
+ X
ORGANELLE J
A
product
A
product
+ X
RELOCATION
ORGANELLE K

Fig. 6. A reactant/product localisation artefact seen in viability-staining live HeLa cells using the colourless MTT Tetrazolium salt as a reactant. Localisation of the coloured MTT Formazan product in the Golgi apparatus (red staining, large white arrow) and lipid droplets (red staining, thin arrows) of a living HeLa cell is as predicted using QSAR modelling. No red staining occurs in mitochondria (green autofluorescence, large empty arrow), nor in the endoplasmic reticulum (unstained), these being the sites of accumulation of the MTT Tetrazolium salt reactant predicted by QSAR modelling. Note that this image uses false colour for MTT Formazan. N: nucleus, nu: nucleolus.

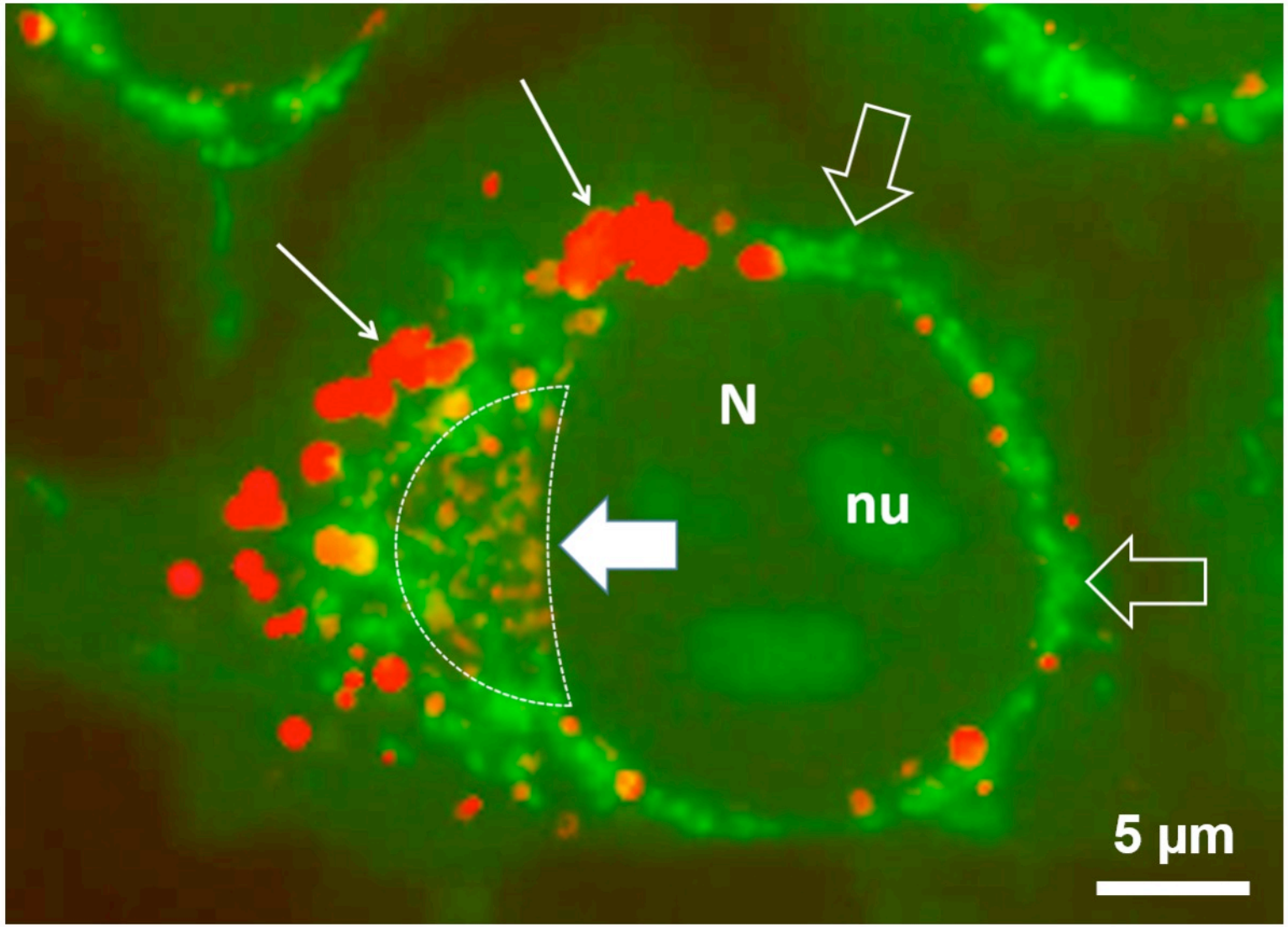

N
nu
5 µm

Fig. 7. Outline of the procedure used to evaluate the likelihood of reactant/product localisation artefacts. Step 1 generates localisation predictions for reactant and product species (see green boxes), and step 2 assesses the significance of the predictions (see orange boxes). For simplicity, this flow chart is restricted to probes applied to cells from an external solution.

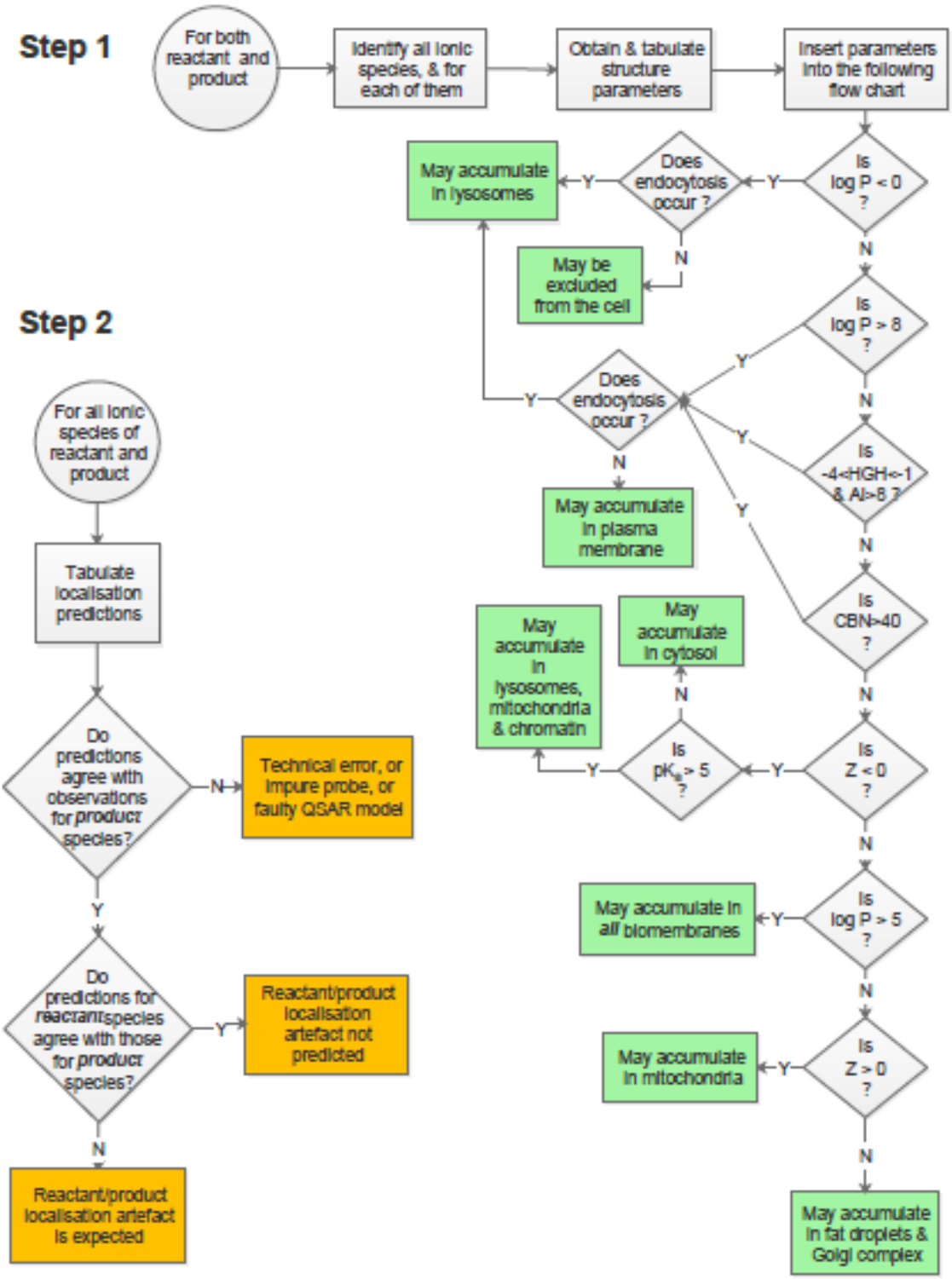

Step 1
For both reactant and product
Identify all ionic species, & for each of them
Obtain & tabulate structure parameters
Insert parameters into the following flow chart
Is log P < 0 ?
Y
Does endocytosis occur ?
Y
May accumulate in lysosomes
N
May be excluded from the cell
N
Is log P > 8 ?
Y
Does endocytosis occur ?
Y
N
May accumulate in plasma membrane
N
Is -4<HGH<-1 & AI>8 ?
Y
N
Is CBN>40 ?
Y
N
Is Z < 0 ?
Y
N
Is pKa > 5 ?
Y
N
May accumulate in lysosomes, mitochondria & chromatin
May accumulate in cytosol
N
Is log P > 5 ?
Y
N
May accumulate in all biomembranes
Is Z > 0 ?
Y
N
May accumulate in mitochondria
May accumulate in fat droplets & Golgi complex
Step 2
For all ionic species of reactant and product
Tabulate localisation predictions
Do predictions agree with observations for product species?
N
Technical error, or impure probe, or faulty QSAR model
Y
Do predictions for reactant species agree with those for product species?
Y
Reactant/product localisation artefact not predicted
N
Reactant/product localisation artefact is expected

Table 1. Some illustrative examples of using fluorescent probes to obtain information concerning living biological systems. Note: "reactive" is used in the inclusive sense, and no dye listed falls into the CI Reactive application class. For more information on the listed dyes see [6], [30] and the Thermo-Fisher Scientific website.

Objectives of the staining process	**Examples of specific information sought**	**Examples of fluorescent probes used for such tasks**	**Is the exemplar probe reactive?**	**Comments**
Identifying biological structures	Presence and location of mitochondria	Rhodamine 123	No	
		MitoTracker Green FM	Yes	Chloromethyl groups bind to protein nucleophiles.
Identifying chemical and biochemical contents	Presence and location of calcium ions	Rhod-2 AM	Yes	Intracellular esterase catalyses de-esterification to generate a chelator which reacts with Ca(II) ions.
	Presence and location of ROS	MitoTracker Red CMXRos	Yes	Reacts with ROS yielding a fluorescent product, whose chloromethyl groups bind to protein nucleophiles.
Assessing biological functions and physicochemical properties	Cell viability	Fluorescein Diacetate	Yes	Intracellular esterase catalyses de-esterification to generate fluorescent product.
	The pH of a cell	Carboxy-SNARF 1	No	
		Diacetyl derivative of 2,3-dicyano-1,4-dihydroxy benzene	Yes	Intracellular esterase catalyses de-esterification to generate a pH indicator.

	The viscosity of a cell region	Lyso-V	No	Lysosomally localised, see [31].
Assessing the location, and changes in location, of cells or organelles	Tracing neuronal pathways within the brain	Fluorogold	No	
		CellTracker CM DiI	Yes	Chloromethyl groups bind to protein nucleophiles.
	Tracking movement of cells	PKH 26	No	
		CellTracker Blue CMAC	Yes	Chloromethyl groups bind to protein nucleophiles.

Table 2. Pay-offs of reactivity for different dye classes in textile dyeing, and for different probe types in investigations of living cells and tissues. Note: 'targets' are the fibre in textile dyeing, and a cell or cell component when using fluorescent probes; occurrence of pay-offs are indicated by tick marks (✓); na indicates not applicable.

Examples of textile dye class or florescent probe type	**Pay-offs provided by reactivity**		
	Facilitation of, application to, or entry into, the target	Increased retention within or adjacent to the target	Identifying the presence of the target
CI dye application classes used for colouration of textile fibres			
Azoic	✓	✓	na
Mordant		✓	na
Reactive		✓	na
Sulphur	✓	✓	na
Vat	✓	✓	na
Fluorescent probes used to investigate live cells and tissues			
AM esters of metal ion probes	✓		✓
Cell tracers/markers		✓	✓
Chemical sensors for H_2S, NO etc			✓
'Fixable' organelle probes		✓	✓
Reduced-dye organelle probes	✓		✓

Table 3. How differences in physicochemical properties influence cellular localisation of MTT Tetrazolium and MTT Formazan, the reactant and product species of a reactive probe. Z, AI and log P are respectively the electric charge, the amphiphilicity index (a measure of the size of the lipophilic domain) and the log of the octanol-water partition coefficient (a measure of overall lipophilicity). The QSAR models providing the predictions of accumulation sites are summarised in [10].

Compound	**Physicochemical properties**			**Predicted site of accumulation**
	Z	**AI**	**Log P**	
Reactant: MTT Tetrazolium	1+	3.0	2.0	Endoplasmic reticulum, mitochondria
Product: MTT Formazan	0	0	4.9	Fat droplets, Golgi complex