Photocontrolling Microtubule Dynamics with Photoswitchable Chemical Reagents

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Summary

Microtubule dynamics can be inhibited with sub-second temporal resolution and cellular-scale spatial resolution, by using precise illuminations to optically pattern where and when photoswitchable microtubule-inhibiting chemical reagents exert their latent bioactivity. The recently-available reagents (SBTub, PST, STEpo, AzTax, PHTub) now enable researchers to use light to reversibly modulate microtubule-dependent processes in eukaryotes, in 2D and 3D cell culture as well as *in vivo*, across a variety of model organisms: with applications in fields from cargo transport to cell migration, cell division, and embryonic development.

However, a wide knowledge gap has remained in the literature which has blocked further translation of these and many other photopharmaceuticals. No generally-applicable procedures or workflows to establish biological assays using photopharmaceuticals have been published. Vital information - including methods to perform assay benchmarking for photoconversion and bioactivity limits, testing for differential isomer solubility, proving the retention of mechanism of action in cells, and estimating the onset of phototoxicity - has either simply not been formalised in the literature, or has remained buried in diverse reports without being unified and codified even within the organic chemistry community, let alone for chemical biologist, microscopist and biologist "user" communities. Accordingly, the rate of adoption of photopharmaceutical tools (beyond the original chemical developers of the tools) has remained very low, which continues to restrain the progress and the impact of photopharmacology.

Here we report a robust four-step assay establishment and cross-checking procedure, to optimise assay parameters for achieving reliable photocontrol over microtubule dynamics, that is also applicable to diverse families of photoswitchable inhibitors beyond the microtubule context. This procedure also controls for many of the most common sources of irreproducibility, and includes numerous troubleshooting steps. We furthermore supply an introduction containing the most relevant information for microscopists and biologists, to explain the theory of small molecule photoswitching; show the unique features, usage requirements, and limitations that photoswitchable chemical reagents have; and evaluate the specific performance features of the major classes of photoswitchable microtubule inhibitors, highlighting their properties that suit them to different applications.

The generally-applicable workflows that we present allow establishing cellular assays that optically control microtubule dynamics in a temporally reversible fashion, with spatial specificity down to a single selected cell within a field of view. These workflows and methods also should be directly applicable to photoswitchable chemical reagents against diverse other protein targets, as well as to advanced uses of photopharmacology in 3D cell culture and *in vivo*. They thus represent an important bridge towards reaching the high-value biological applications that photopharmacology has long promised.

Keywords: cytoskeleton; microtubule dynamics; tubulin polymerisation inhibitor; photopharmacology; optogenetics; photopharmaceutical; optical control; photocontrol; antimitotic; cell division; cell migration; development; photoswitch.

1. Introduction

Modulating microtubule (MT) dynamics and network integrity with suitably high spatiotemporal precision has unique potential to enable a broad range of studies within fields from signalling and cargo trafficking, to cell shape maintenance, migration and division. Tubulin-inhibiting small molecule drugs ("antimitotics") such as colchicine or taxol that have no spatiotemporal precision are nevertheless widely used to interfere with MT dynamics and depolymerise or stabilise MTs; and they have had enormous impact on fields from cytology and development to neuroscience and cancer therapy [1] (see **Note 1**). Photoswitchable small molecule microtubule-inhibiting reagents that do feature spatiotemporal precision have been developed since 2014, and offer exciting potential for diverse research applications [2].

Photoswitchable small molecule reagents, known as "photopharmaceuticals", have a unique combination of performance features, and offer many of the advantages of optogenetics and photouncaging, with only a few drawbacks. These reagents are simple to use in practice and are easy to transition between model systems, from 2D to 3D cell culture, explants, and embryos. Many investigators have established microtubule photocontrol assays in their own biological systems, using diverse instrumentation, achieving extremely precise control over MT-dependent processes in the short and long term: even as part of an undergraduate biology practical course (Dr. Isabell Smyrek, Institute of Cell Biology and Neuroscience, University of Frankfurt). However, the behaviour of photoswitchable small molecule reagents is significantly different from that of other optical systems. In our experience it is most efficient to first review the theoretical differences, and then to consciously establish assay settings from scratch, rather than reusing protocols from optogenetics or photobleaching experiments.

To our knowledge there are no general guides for biologists to establish or troubleshoot cell biology assays relying on small molecule photoswitchable reagents. Nor are there concise summaries of the relevant theoretical issues written for a biological audience. The current reviews on photopharmaceuticals either are written for synthetic chemists, or else they focus on potential future applications to medicinal chemistry [3–6]. The most relevant prior works are two short user guides and discussions for two specific MT photoswitches, PSTs (see "Supporting Information: Appendices" in [2] (20 pages)) and SBTubs (see "Supporting Information: Practical User Guide" in [7] (6 pages)). There, the interested reader will find much generally-applicable detail to complement the theory given here, and to help evaluate the assay settings used in primary reports of the various MT-inhibiting photoswitches.

We here focus towards establishing one type of assay: temporally reversible, cell-specific inhibition of microtubule dynamics, in a single targeted cell within a field of view, using either taxol- or colchicine-like photoswitchable reagents. The degree of photocontrol over MT dynamics that these assays permit is typically sufficient to allow optical targeting of MT network depolymerisation, mitotic arrest, arrest of migration, and even cell death; and the workflow we follow will also allow the experimenter to apply this level of control to multicellular regions, in 2D or 3D systems including in early embryos, by adapting the protocols accordingly.

We will introduce only those aspects of photoswitching that we feel are most relevant to biological use. We also limit our discussion to those photopharmaceuticals that have been used to control cellular MT biology. The reader is referred to chemical reviews for general information about the scope of photoswitch chemistry.

1.1 What are photopharmaceuticals, and how does *E*↔*Z* photoswitching operate them

Photopharmaceuticals are small molecules that undergo a reversible structural change or "isomerisation" upon absorbing light. These reagents consist of a chemical photoswitch fused into the structure of a "parent" pharmaceutical drug type (e.g. colchicine, taxol, epothilone, etc as MT inhibitors; see **Note 2**). Photopharmaceuticals have many advantages compared to photouncageable drugs (reviewed in [7], see also **Note 1**); however even well-designed photopharmaceuticals often have 10- to 100-fold losses of potency compared to the parent drug, due to the need to include the photoswitch (SBTub2M is a rare exception that retains high potency; see also **Note 2**). In this chapter we will only present MT photopharmaceuticals that we consider thoroughly mechanistically validated (see **Note 3**).

There are several classes of photoswitch that can be used in photopharmaceuticals, and each has distinct biological and chemical properties (see **Note 4**). On the single-molecule level, a photoswitch has two states (or *isomers*), called *E* (or *trans*), and *Z* (or *cis*) (see **Note 2**). The "ideal" photopharmaceutical designs are reagents where (i) one isomer is bioactive while the other is not active at all; and (ii) two different wavelengths of light λ_1 and λ_2 can be used to isomerise all molecules completely first to one form, and then to the other, respectively (**Fig 1a**). With such an ideal, a biological sample could be treated with the photopharmaceutical in its inactive isomer, and then "photopatterned" by illuminating one zone continuously with λ_1 and a second zone continuously with λ_2, so that MT dynamics are strongly inhibited in zone 1, while zone 2 (and non-illuminated cells) are kept entirely free of inhibition, despite diffusion (**Fig 1a**). However, this ideal has never been achieved (see **Note 5**), it may even be chemically impossible. Still, highly effective results can be achieved by first gaining a realistic picture of the performance and possibilities of chemical photoswitching, and then designing assays accordingly.

1.2 Photopharmaceuticals: "dark" state, "dark conditions", and "relaxation"

Most photopharmaceuticals are exclusively present as one isomer in their "dark state" (before receiving any illumination). For the MT reagents we describe, the dark state means that a compound is completely present as the *E* isomer. As long as a stock has been prepared from a pure dark state, and stored and handled under "dark conditions" (see **Note 6**) it will be 100% *E* isomer upon use. Most photoswitches also "relax back" from any *Z:E* ratio until they return to the dark state, a process known as "spontaneous" or "thermal" relaxation: but for MT photopharmaceuticals this is usually much slower than biological assay timescales and can be ignored (see **Note 7**).

1.3 Operating photopharmaceuticals 1: the wavelength of photoisomerisation light sets the *Z:E* ratio

Complete photoisomerisation from *E* to *Z* and then back again from *Z* to *E* isomers is not possible with any known photoswitch. Photoisomerisation using a particular wavelength can start from any *Z:E* ratio, but will eventually convert the sample to a new *partially isomerised* equilibrium state (called a "PSS") where the *Z:E* ratio of this state depends only on the choice of the illuminating wavelength. Thus, the degree of isomerisation at PSS cannot be "improved" just by applying more light at the same wavelength. It is a common mistake to keep applying light to the sample in the hope that an effect will eventually be seen (which is usually futile; see **Note 8**).

The first task for the biologist is to choose the "best practical" photoisomerisation wavelengths -- those available on the setup, that give the most complete photoswitching -- by examining what are the *Z:E* ratios at PSS at those available wavelengths (see **Note 9**). We use $\Psi(\lambda)$ to denote the fraction of *Z* isomer established in a population by illumination at wavelength λ reaching PSS. For example, using fixed laser lines, reagents that contain an azobenzene photoswitch (e.g. PST reagents and AzTax3MP; see **Note 3**), typically have a best practical λ_1 (enriching the *Z* isomer) at either 355 or 405 nm, that converts the photopharmaceutical to a 85:15 *Z:E* isomer ratio; while the best practical wavelength λ_2 for "isomerising back" (enriching the *E* isomer) is typically 488 or 514 nm reaching a 20:80 *Z:E* ratio. Thus if zone 1 in a sample is illuminated with a ROI at 405 nm and zone 2 at 514 nm, $\Psi(405)=0.85$ and $\Psi(514)=0.20$ are reached, respectively (**Fig 1b**). These ratios are currently considered as very good.

Note that within the biologically compatible wavelength range (>340 nm), some photoswitch types can essentially only be photoisomerised "in one direction". Examples include the SBT and ST photoswitches (as in SBTub and STEpo reagents) where the best λ_1 (typically 360-400 nm) may convert the SBT/ST photoswitch to a 85:15 *Z:E* ratio. Deep UV would be needed to photoisomerise these reagents "back" towards significant *E* fractions. For such reagents to achieve reversible MT patterning, the *Z*-isomer of the reagent within a zone or a cell must instead diffuse out of the cell (though similar to photouncaged reagents, this still has numerous advantages, discussed in [7]).

Although "technical best" wavelengths for isomerisation are sometimes quoted, it is much more helpful to have a table or chart that describes $\Psi(\lambda)$, the fraction of *Z* of the photoswitch as a function of wavelength (**Fig 1c**). For example, this helps avoid a common mistake, of imaging a photoswitch-treated sample using a wavelength that the

photoswitch itself responds to (see **Note 10**). Few photopharmaceuticals other than SBT and ST are GFP-compatible (some special azobenzenes are, but not PST or AzTax). Even RFP excitation at 561 nm typically isomerises azobenzenes (e.g. **Fig 1b** shows 561 nm imaging causing a 9:91 *Z:E* PSS, typical for PST and AzTax) (see **Note 10**).

Finally, it is helpful to estimate a photopharmaceutical's *relative speeds of photoresponse* to any given wavelengths. We have proposed a "photoswitching efficiency" metric E(λ), a normalised combination of the isomers' absorption coefficients at λ [2] (**Fig 1c**: plotted on the right, logarithmic, axis). Here, as E(405)/E(514) = 30, this shows that the PST's photoresponse to λ_2 = 514 nm is approx. 30-fold less sensitive than to λ_1 = 405 nm. Therefore, if identical photon fluxes (intensity per pixel multiplied by time per pixel) were used for isomerisations at 405 and 514 nm, the *Z*→*E* isomerisation at 514 nm would be much slower than *E*→*Z* isomerisation at 405 nm (shown in **Fig 1d**); higher intensities or pixel dwell times during 514 nm illumination would help compensate for slower photoresponse. E(λ) is also useful to predict what happens when two or more wavelengths are applied to a sample at once. For example, a common mistake is to use "white light" for focussing on a sample. If this light contains all wavelengths from 400-700 nm, a photopharmaceutical will isomerise most strongly from those wavelengths with the highest E(λ). In this example (**Fig 1c**), that means 400-450 nm, so stimulating isomerisation towards the corresponding Ψ(λ) (on average ca. 80% *Z*, **Fig 1c**). For the PST this is disastrous, since MTs in all cells in the field of view would then be inhibited before the experiment has begun. We recommend using light above 600 nm for focussing where possible.

1.4 Operating photopharmaceuticals 2: the *Z:E* ratio and total concentration control the bioactivity

Having chosen λ_1, λ_2, and the imaging wavelength, the *Z:E* ratios in different zones should be calculated (e.g. zone 1, zone 2, or imaging-only areas in **Fig 1b**: note that these can be spatially *or* temporally resolved zones). In our example, zone 1 will have 85% of the photopharmaceutical as *Z*, zone 2 will have 20% as *Z*, and imaging-only areas will have 9% as *Z*. If the rough cellular concentrations needed for bioactivity are known, an appropriate working concentration can now be estimated. In the case of PST-1/PST-2, the *E* isomer is completely inactive up to 50 μM; in short-term imaging experiments, ca. 2 μM is the threshold for onset of bioactivity of the *Z* isomer (MT plus tips move slower, cover shorter distances, and are less dense), while depending on the readout and quantification, ca. 7.5 μM of the *Z* isomer causes the maximal possible inhibition of MT polymerisation (**Fig 1d**, dotted limit lines).

To attempt fully-uninhibited/fully-inhibited photoswitching using 514/405 nm photoswitching, a total concentration of 10 μM PST-1 could therefore be applied. Phases of 405 nm should raise the concentration of *Z* isomer to 85% of 10 μM = 8.5 μM (fully inhibited), while phases of 514 nm should decrease the concentration of *Z* isomer to 20% of 10 μM = 2 μM (borderline for inhibition); while imaging at 561 nm should slowly isomerise the sample to 0.9 μM *Z* (uninhibited) (**Fig 1d**). Note that the speeds of photoresponse are different for each phase, according to the photoswitching efficiency E(λ) (see section **1.3**). **Fig 1e** [2] shows the experimental result of a similar assay. The initial phase at 561 nm (imaging only, baseline) does not inhibit MT dynamics. Then phases of 514 nm and 405 nm are alternated, and cellular MT dynamics are reversibly photoswitched between borderline inhibition and drastic inhibition respectively (see section **1.6** for a discussion of the decrease in dynamics over time).

Note however, if the sample had instead been treated with 20 μM of the *E*-isomer photopharmaceutical (perhaps in the hope of seeing still stronger inhibition during λ_1 phases), then λ_2-illumination would have produced 4 μM *Z* isomer: so that the cells would have experienced substantial undesired MT inhibition throughout the λ_2 phases as well: so the degree of photoswitching would have been lower. This shows that to understand how a sample will react to photoswitching, it is vital to track the fractions of *E* and *Z* isomers within "populations" of photoswitch molecules at distinct spatial and temporal zones, and to calculate the concentrations of the bioactive isomer(s) in each population based on the total concentration applied. **Fig 1f** shows experimental results of a longterm cell viability assay which reveals just how predictive this fraction tracking can be. Varying the illumination wavelength, changes the fraction of bioactive *Z* isomer in a PST-treated sample (compare the Ψ(λ) values in **Fig 1c**), and therefore shifts the dose-response curves in an assay by an entirely predictable amount [2].

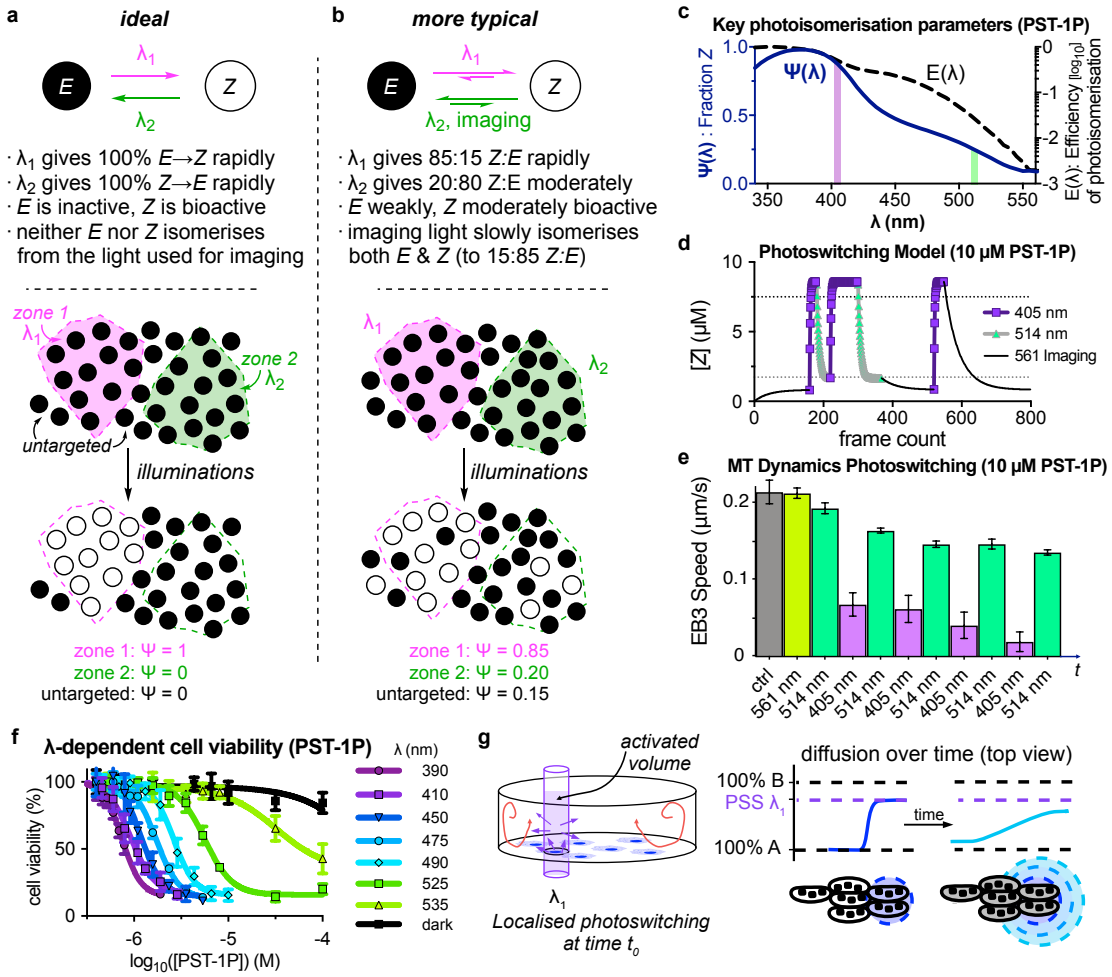

Fig. 1 Photoswitching Theory and Practice. (a-b) "ideal" and realistic situations of photopatterning a photopharmaceutical, with panel b showing the partial completeness of photoisomerisations, and the response to imaging wavelengths. **(c)** Experimental *Z*-isomer fraction Ψ(λ) [linear axis at left] and photoswitching efficiency parameter E(λ) [log axis at right] for representative azobenzene MT photopharmaceutical PST-1P. These functions describe the photoresponse of a photopharmaceutical to any given wavelength of light. **(d)** Model for typical phases of photoswitching, changing the concentration of *Z* isomer within a sample of PST-1P over time. **(e)** Experimental data on photoswitching MT polymerisation dynamics over time, using illumination phases as in panel d (EB3 is an MT plus tip marker, see Methods). **(f)** Experimental data showing that the wavelength-dependent *Z*-isomer fraction Ψ(λ) determines the cellular bioactivity dose-response profile of PST-1P. **(g)** Photoswitching selected cells by transmitted light also photoswitches compound in the optical path volume within the media, usually giving background activity. Light scattering and numerical aperture > 1 increase the illuminated volume and the background. Diffusion counteracts the spatial localisation of photoisomer patterning (or equivalently, re-equilibrates a photopatterned cell against the medium). Diffusional reversibility is greatly accelerated by convection motion in the medium (e.g. heated stage), and is slowed down when cells are shielded from convection currents by matrix embedding or a 3D environment.

1.5 Operating photopharmaceuticals 3: photoisomers diffuse rapidly through cells, and to/from the medium

Photoswitchable inhibitors freely enter and leave cells, crossing the plasma membrane on a timescale of tens of seconds. Both the *E* and *Z* isomers cross membranes, both before *and after* photoswitching, and in both inwards and outwards directions: i.e. they are always *dynamically exchanging* to the bath medium and neighbouring cells. If cells are grown on a coating, in a gel or 3D culture, or if a tissue explant or embryo is being treated, then these barriers will slow down the drug access to cells far from the surface as well as slowing the drug's diffusion back out to the medium, but diffusional exchange is always present. Though this exchange does not prevent using localised

photoswitching to precisely target MT inhibition, anticipating both pre- and post-photoswitching diffusional exchange is important for designing and interpreting assays. For example, if an embryo is "loaded" by being bathed in photoswitch solution, then removed, washed and embedded for imaging, the loaded compound will immediately start washing out of the animal (minutes scale). Washout in a 2D cell culture is much faster and can be complete in < 60 s (see **Note 11**). In both cases, attempting photoswitching after washing can be complex, since only an unknown and constantly decreasing amount of the photopharmaceutical remains in the system.

Convection currents in dishes are particularly important for fast compound exchange in and out of cells when these are kept in 2D cell culture without a coating / matrix. These currents (orange swirls, **Fig 1g**) arise because of slight temperature differences in the solution (e.g. heated stage) and when they contact cell surfaces, rapid mixing occurs which is beneficial for fast temporal reversibility of MT inhibition. Alternatively, embedding in a gel or matrix prevents these currents from contacting the cell surface, so embedded systems have slower exchange (see **Note 11**), and so can have more persistent spatial patterning of MT inhibition.

It is important to realise that photoswitchable small molecule inhibitors also diffuse orders of magnitude faster *within* cells than proteins do. As an estimate, a subcellularly-patterned photopharmaceutical in an ordinary cell has probably equilibrated throughout the entire cell within ca. 5 seconds. Therefore it is usually futile to attempt subcellular patterning of MT *network* de/stabilisation using these reagents because those structure changes typically require minutes (one exception are MTs in mitotic cells, which can be very rapidly depolymerised: private communications, Wallis Nahaboo, University of Brussels and Jenny Taylor / Clemens Cabernard, University of Washington). We have had moderate results so far with subcellular patterning of MT *dynamics*. It seems that this can be achieved to some degree with two-colour photoswitching using PST reagents: careful tuning of concentration and (fast) photopatterning repetition rate allows zones separated by ca. 5-10 μm to be kept differently photopatterned (inhibited vs noninhibited) over a timescale of minutes [8]. Unusual cellular geometries such as neurons, giant cells and very early-stage embryos make this task easier as subcellular zones can be larger than compound diffusion lengths. However: in general, subcellular patterning is difficult with photopharmaceuticals, and expectations should be lower than for optogenetics or protein FRAP (slower-diffusing proteins give more persistent patterning).

1.6 Operating photopharmaceuticals 4: always consider the full light path of all light applied

Unlike photoresponsive or fluorescent proteins, photopharmaceuticals are present *both* in the cell *and* in the culture medium, throughout an experiment. While the compound is photoswitched in target cells, it is unavoidably also photoswitched in the medium since the transmitted light beam passes through selected cells or fields of view then travels through the rest of the medium above that cell (optical path volume; see **Fig 1g**). This photoswitched compound in the medium is free to spread rapidly by diffusion and convection throughout the sample, also entering non-targeted cells: creating increasing levels of background activity over time (compare **Fig 1e**). Light scattering from inhomogeneities in 3D explants, animals, or surfaces, creates a larger optical path volume; higher numerical apertures might also somewhat contribute due to their flatter light cone angles. For good results this background should be minimised. Primarily this means minimising the photon flux per photoactivation phase (λ_1) to the minimum of what is necessary. An elegant possibility, although we are unaware of this having been performed before, would be to use TIRF illumination for photoswitching, to eliminate the optical path volume above the cell; this should theoretically allow several hundred times more photoswitching cycles than transmitted light photoswitching.

1.7 Choosing inhibitors for photoswitching-based optical control of MTs

Photopharmaceuticals for MTs have enabled optical control over a range of MT-dependent biological processes, in settings from 2D cellular studies to applications in organoids, early-stage, and later-stage embryos (see **Note 13**).

Several families of photopharmaceutical MT destabilisers have been developed that are functionally analogous to colchicine (or equivalent colchicinoids such as nocoazole, colcemid, combretastatin, etc). Advantageously, the SBTub and PST families are truly inactive in their dark state, with good (PST) to outstanding (SBTub) potency as

their *Z*-isomers (photoactivated state) (see **Note 2**). This high activity ratio offers very clean assay setups, without background activity. SBT-based colchicinoid SBTubs are the photopharmaceuticals of choice for all assays except (1) where actively bidirectional (two-colour) photoswitching of MT dynamics inhibition is needed (i.e. where unidirectional *E*→*Z* photoswitching, followed by diffusional or convectional reversibility, is not sufficient; in our experience this is seldom): in which case PSTs are the reagent of choice; or (2) where MT stabilisation is required as a mechanism of action: in which case STEpo or AzTax are the reagents of choice (see below).

The most powerful SBTubs are the highly potent cosolvent-requiring SBTub2M, and slightly less potent but buffer-soluble SBTubA4P [9]. These replace first generation SBTub3 and SBTub3P [7]. They are also the best reagents for *in vivo* studies, due to their extreme metabolic stability and their excellent potency, their solubility, and rapid permeation into cells. SBTs and the related STs are photoisomerised efficiently at 355-405 nm, but have the unique feature of being fully GFP/YFP-compatible (non-responsive to ≥485 nm) unlike other photoswitches; this leaves a valuable additional imaging channel free. They seem to diffuse rapidly, with a diffusional recovery halftime of 20 s in 2D cell culture. Azobenzene-based colchicinoid PSTs [2] (e.g. PST-1, PST-2, PST-1P, PST-2S) are the reagents of choice only where actively bidirectional (two-colour) photoswitching of MT dynamics inhibition is needed. PSTs are ca. 10-fold less potent than SBTubs, and can be metabolised, which may pose problems in long-term *in vivo* assays. Typical PST photoswitching wavelengths and imaging respose were given in **section 1.3**. For citations, and considerations for using SBTubs and PSTs *in vivo,* see **Note 13.** For HTI-based colchicinoids see **Note 14.**

MT stabilisers like epothilone and taxol have enabled a variety of research and therapeutic applications which are inaccessible to destabilisers due to their differing pharmacology, stoichiometry, and spectrum of biological effects [10–12]. Accordingly, two classes of photopharmaceutical MT stabilisers have also been developed, STEpo and AzTax. Note that both classes have significant residual bioactivity as the *E* isomers before photoswitching, so concentration tuning is imperative, and is more demanding than with PSTs and SBTubs. ST-based epothilone derivatives, STEpos (best compounds STEpo2 and STEpo1), are high-potency MT stabiliser photopharmaceuticals with similar photoswitching as seen for the SBTubs (unidirectional *E*→*Z* photoswitching at 360-405 nm, and GFP/YFP-compatibility); they are likely to have similar metabolic robustness too. STEpos have only been demonstrated in cell-free and 2D cell culture studies so far. They appear to have rather slow diffusional reversibility (halftime >5 min), which may be related to their binding potency and their mechanism of action, and this may be beneficial for long-term localised inhibition [13]. Azobenzene-based taxol derivatives, AzTax (best compound AzTax3MP), are moderate-potency MT stabiliser photopharmaceuticals which have moderate diffusional reversibility (halftime ca. 70 s) as well as moderate ability to apply subcellularly-localised effects in large neurons [14]; however their combination of low solubility and low potency disfavours applications beyond 2D cell culture. Although two-colour photoswitching should technically be possible with AzTax, in our experience it was unsuccessful. Therefore STEpo1/STEpo2 appear the reagents of choice for MT stabilisation assays, except if faster diffusional reversibility is absolutely required: in which case the reagent of choice is currently AzTax3MP.

When choosing a photopharmaceutical for any experiment, it is beneficial to ensure it has efficient photoswitching and high potency. For cell-localized persistent inhibition, a slow-recovering compound should be used (e.g. STEpo); for cell-localised but rapidly-recovering inhibition, either a rapidly-diffusing compound should be used (e.g. SBTub), or else an efficiently two-colour switchable compound (e.g. PST) (**Table 1**).

Table 1: Overview of photoswitchable MT inhibitors with relevant performance properties for localized photoactivation experiments, and for recovery after photoswitching. Asterisk* denotes that the marked wavelength stimulates photoconversion towards a majority of the dark state isomer.

Reagent	Class	Photoswitch class	GFP-compatible	diffusion rate ≈ MT dynamics recovery rate	Efficiency of photoconversion	Localized activation	Solubility
SBTub2M SBTubA4[P]	Colchicine	SBT	Yes	Fast	High (360/405)	Poor	[Prodrug: no cosolvent needed] Drug: Moderate

PST-1[P] PST-2[S]	Colchicine	azo	No	Fast	High (360/405) Moderate (*514)	Good	[Prodrug: no cosolvent needed] Drug: Moderate
AzTax3MP	Taxol	azo	No	Moderate	Moderate (405) Low (*514)	Moderate	Poor
STEpo2	Epothi-lone	ST	Yes	Slow	High (360) Low (405)	Moderate	Moderate
PHTub-7	Colchicine	HTI	No	Slow	Low (440) Moderate (490) High (*514)	Good	Poor

In conclusion, SBTub2M and SBTubA4P are excellent general-purpose tools for light-dependently blocking MT dynamics with spatiotemporal precision across nearly all assays. In 2D cell culture however, SBTub, PST, and AzTax are all easy to use in the basic assays of *temporally precise, and optionally also cell-precise, repeated and reversible blockades of MT dynamics* that we will work towards establishing (see **Fig 1e**). We now outline workflows to establish these basic assay types in 2D cell culture.

2. Materials

- Microscope fitted with heated incubation chamber with CO_2, best with 360 or 405 nm laser line for photoactivation, else 440 nm
- *Helpful*: Light proof cover for imaging chamber (can also be a piece of matte black plastic)
- Red light lamp for working "dark"
- Light proof brown glass vials or Eppendorf tubes for "dark" stocks
- Clear colourless glass vials or Eppendorf tubes for "lit" stocks
- DMSO (if required for compound solubility) (see **Note 12**)
- Cell medium
- Photoswitchable microtubule inhibitor (typically 1-10 mM stock solution in DMSO) adapted to the photoactivation laser line. 360 or 405 nm are appropriate for SBTub, STEpo, AzTax, and PST (AzTax and PST slightly better at 360-380, SBTub better at 405); 440 laser is appropriate for PST, AzTax or PHTub only).
- Reference inhibitor from the same drug class as the photopharmaceutical MT inhibitor, ideally of similar potency (e.g. colcemid, docetaxel, epothilone D, etc).
- *Helpful*: Appropriate permutation control compound to the photopharmaceutical (see **Note 3**).
- *Helpful*: Commercial LED at the same wavelength as your photoactivation line (e.g. 365 or 405 nm)

3. Workflow

3.1 Establish Imaging Conditions and Test Reference Inhibitor

1. Plate cells on coverslips that fit inside live cell imaging chamber or dishes compatible with live cell imaging. Transfect cells with fluorescently tagged EB1 or EB3 construct (see **Note 15**) using cell-type appropriate transfection protocol.
2. Prepare incubation chamber on microscope at 37 °C with 5% CO_2.
3. Place cells in imaging chamber or dish inside incubation chamber on microscope. Image EB comets every 2-4 seconds for 5-10 minutes, using different laser power and exposure settings. Analyze movies using ComDet plugin (see **Note 16**) to obtain comet counts per frame. Becoming familiar with expected baseline levels of comet density and speed, and compromising for good detection but low bleaching rate, will be crucial for all coming steps. Based on analysis select laser power and exposure settings with minimal comet bleaching over time (see **Figure 2** and **Note 17**). Depending

on what the final assay readout should be, also decide upon a suitable frame rate: the lower the frame rate, the less bleaching, the more reliable an experiment will be, and the longer it can be run.

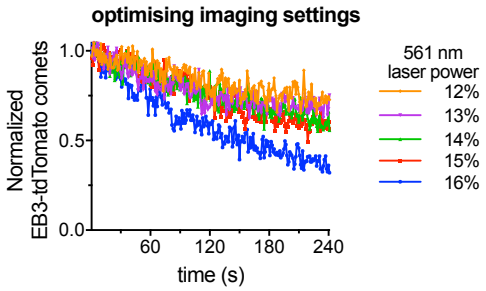

Figure 2: Optimization of imaging laser power. 561 nm laser power percentages (indicated) tuned during imaging EB3-tdTomato comets on a particular setup. 13% was selected as optimal: low bleaching, high signal.

4. *If the photopharmaceutical is water soluble (eg. SBTubA4P, PST-1P, PST-2S) ignore step 4.* Test the maximum cell tolerance to cosolvent by applying necessary concentrations (e.g. 0.5-2% DMSO; see **Note 12**) diluted in media to cells. Image EB comets. If cells start to round up, or EB comets stop, or EB comet counts decrease much faster than normal, this indicates that cells are not tolerant of DMSO at this concentration. The minimum necessary cosolvent is often ca. 1% DMSO per 10 μM of photopharmaceutical (but see **Notes 11-12**). If the tolerated DMSO concentration is not enough to support the minimum reagent working concentration established in step 10, switch to a water-soluble reagent instead.

5. Next, test cell tolerance to the ultraviolet (UV) laser line that is available to you (360-405 nm). Image EB comets while applying UV light at different intensities and exposure times, if possible using a cell-localised ROI with a control cell also in the field of view (see **Note 18**). Analyze comet counts to identify an maximum total applied energy of UV light which, in combination with DMSO (if applicable), is not toxic to cells. This will limit the number of UV photoactivation frames/ROIs that can be applied in an experiment.

6. Lastly, test a reference inhibitor that has the same binding site as the photoswitchable reagent, to confirm that EB comets are indeed suppressed, and to establish expectations for the maximum possible size of effect. E.g. use nocodazole or colchicine as a reference inhibitor for SBTub or PST reagents; use docetaxel or paclitaxel as a reference inhibitor for AzTax reagents; use epothilone D for STEpo. The photopharmaceutical will never be more potent than the reference inhibitor, often it will be substantially less potent: so without a clear readout from the reference inhibitor, the assay should be stopped and reconsidered.

3.2 Find Working Concentration Limits, and Limiting Performance, of the Photoswitchable Reagent

7. Make the room completely dark, with the exception of red lights (**Fig 3a**; see **Note 6**).

8. Maximum working concentration: Dilute the dark state of drug in the following order: DMSO (if applicable) then drug stock, then pre-warmed cell media (see **Note 11**, **12**), then apply this stock to cells. Cover imaging chamber with light proof cover (see **Fig 3b-c**). Caution - use red or green light for focusing on the sample (see **section 1.3**). Image EB comets without UV and check that dark state drug alone does not already slow or decrease EB comets. Vary the drug concentration to find the maximum working concentration: where the dark state has no significant effect on EB comets. (If your compound stock was already exposed to light, incubate the stock overnight at 60 °C to relax it back to the dark state before starting Step 8; see also **Note 7**).

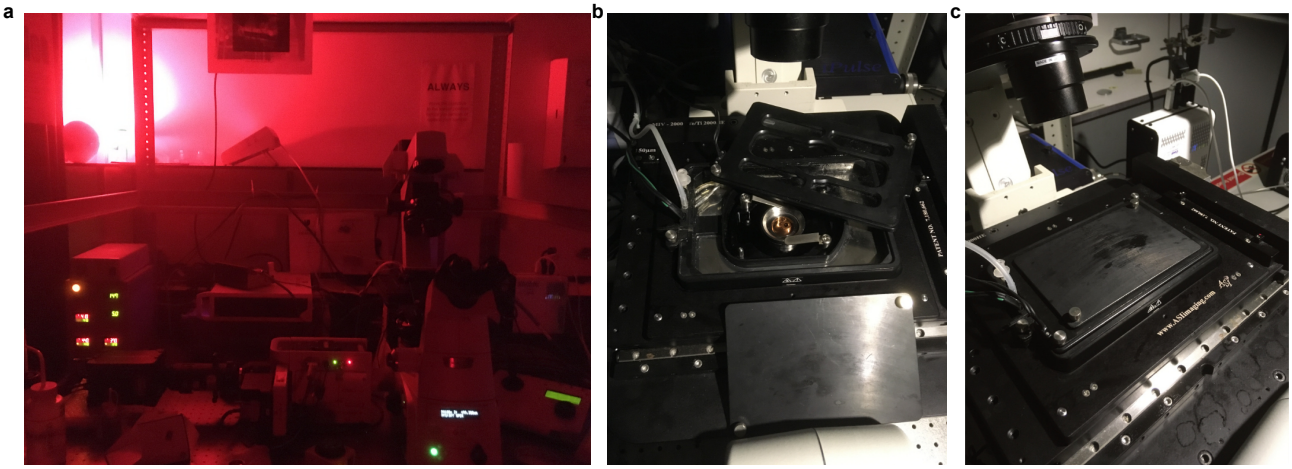

Figure 3: Experimental Setup. (a) Redlight conditions are used to illuminate the room whenever working with switchable compounds; **(b)** stage fitted with heated, humidified incubation chamber with CO_2 and lightproof cover (removed); **(c)** chamber closed with lightproof cover attached.

9. Maximum reagent effect: In the clear colourless glass vial or the clear Eppi, shine a similar photoactivation wavelength as you will use on the microscope (e.g. a 405 nm LED, or a monochromator, or a UV lamp) onto 40 µL of 1 mM DMSO stock of the photopharmaceutical for 1 min, holding the stock as close as possible to the light source. This photoactivates the "lit" DMSO stock. Apply the lit drug to cells at the maximum working concentration established in step 8 and image (see Step 8 for dilution order). This benchmarks the maximum effect that will be achieved by total photoactivation of the reagent *in situ*. If not enough effect on EB comets is visible, wait a little and re-image (especially if not working in 2D culture); if effect is still not seen, then the compound will not be able to work in the assay (troubleshooting: (i) use a glass vial for the illumination, (ii) recheck the maximum working concentration from step 8, (iii) check cosolvent to ensure solubility, (iv) switch to a more potent reagent).

10. Minimum working concentration: Titrate the lit concentration down by factors of 2 to find the minimum working concentration: where the *maximally-activated* state has the lowest acceptable inhibition of EB comets.
    The assay working concentrations should from now on be between the minimum and maximum. It is usually best for cell culture assays that require recovery, to work at no more than ca. 2-3 times the minimum working concentration, so that background activation does not compromise the assay; this is especially important when the imaging light itself isomerises the photoswitch (see section **1.4**: SBTub and STEpo escape this problem, but PST and AzTax do not). For *in vivo* assays it may be best to work close to the maximum working concentration, so that minimal photoactivation results in a strong effect.

3.3 Perform Field Of View Photoswitching at a Working Concentration between the Min and Max

11. Apply dark drug to cells (see Step 8 for dilution order). Photoactivate with 360-405 nm using power settings in the range acceptable for cells (Step 5), typically one frame photoactivation per 3 seconds, full field of view, while imaging EB comets. Titrate drug concentration to be the lowest such that the in situ-activated state has a robust biological effect (**Fig 4a**). Note also the time needed to see inhibition: in **Fig 4a**, the largest decrease in EB3 comets occurs in the first 20 seconds of photoactivation, i.e. at least the next 40 s of photoactivation are dispensible and photoactivation periods can

be reduced to 20 s. Observe cells for recovery after activation (**Fig 4a**: ca. 80 s); if they do not recover, (a) try titrating concentrations down further and (b) reduce the number of photoactivation frames. A permutation control to the photopharmaceutical (see **Note 3**) can be used for optimal verification that e.g. no phototoxicity is operating.

12. If multiple activations and recoveries are important for the assay, take the reduced photoactivation periods, separated by the recovery times, as determined from step 11; then adjust the photon flux during the sequential photoactivation periods (it is easiest to titrate frame exposure time or pixel dwell time, which have a linear relationship to flux, rather than laser power which does not), and the recovery times between photoactivations, until recovery between each period is achieved (**Fig 4b**).

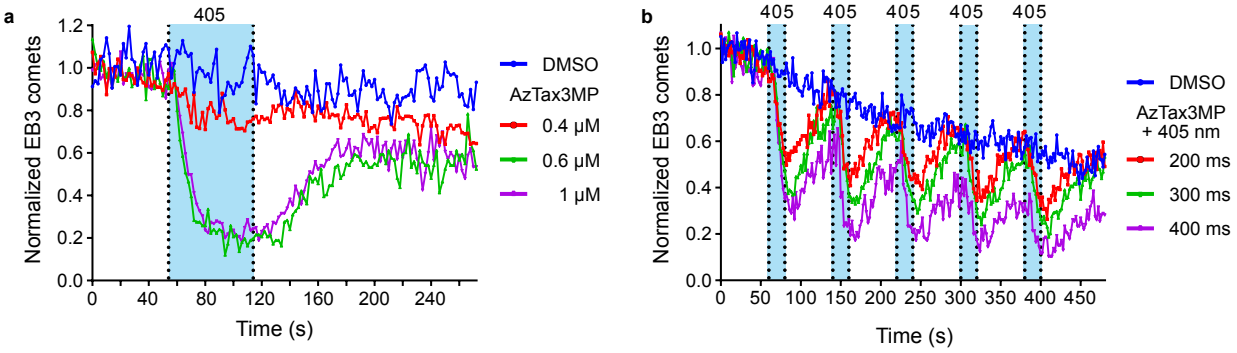

Figure 4: Titrating drug concentration and activation time. (a) *E*-AzTax3MP (dark state) was added to EB3-tdTomato transfected cells and after 60 s baseline acquisition, 405 nm illumination was applied for 1 minute. 600 nM AzTax3MP was determined to be an optimal drug concentration (full inhibition, then recovery). **(b)** different exposure times of 405 nm laser were tested to optimize both microtubule growth inhibition and recovery of microtubule dynamics after inhibition, here 300 ms activation time was found to be optimal as 400 ms did not show full recovery and 200 ms had a smaller reduction in comet counts upon activation.

3.4 Perform Single-Cell Photoswitching

13. To perform temporally and/or two-colour reversible photoswitching in one cell within a field of view without affecting the other visible cells, requires localised photoswitching in an ROI that is usually drawn as only a part of the target cell (typically 10-40% of its surface area, located at the cell centre), either using the laser scanning function, or through a FRAP module. Point-exposure "bleach" functions have also been used, but are probably more disruptive to cells: instead, where possible, apply the same photon flux per pixel for these ROI photoactivations, as used in the whole-field-of-view isomerisation in Steps 11-12. Apply the chosen localised one or two-colour illuminations in ROIs to a target cell, and confirm that the comet bleaching rate is acceptably low. The ideal is to have a second, non-ROI cell in the field of view that can be used as an internal control (see **Note 18**). EB comets from each cell can be analysed separately and normalised to the average of each of their first five imaging frames for proper comparisons. Comet analysis over time of the ROI-cell compared to the non-ROI-cell is essential for this.

14. Apply the dark state photopharmaceutical to cells at the same or optionally up to 50% higher concentration as used in Steps 11-12. Find a field of view with at least two cells with good EB expression levels. Starting with the same photoactivation pixel dwell time settings as in Step 13, titrate the photon flux per pixel in the ROI, and optionally the ROI size or the drug concentration, until ROI-cell-localised comet inhibition, but normal comet dynamics in the non-ROI-cell, are confirmed: comet analysis over time of the ROI-cell compared to the non-ROI-cell is essential for this. We recommend to work at conditions that cause minimal inhibition in the non-ROI-cell, rather than

necessarily maximising the inhibition in the ROI-cell. Re-verify that photobleaching is not problematic with these photoactivation settings; a permutation control to the photopharmaceutical (see **Note 3**) can be used for optimal verification that e.g. no phototoxicity is operating.

4. Notes

Note 1. Other Approaches to Spatiotemporally Specific MT Inhibition. As these drugs suppress all MT-dependent functions without any spatiotemporal specificity [16], recently, several approaches harnessing spatiotemporally-precise applications of light to modulate cytoskeleton dynamics and network architecture on biologically appropriate length and time scales have emerged [17]. Photouncageable antimitotics were the first to be developed and have achieved some key applications [18, 19]; however, they faced several limitations and are no longer commercially available. Optogenetic approaches to manipulate MTs have only been reported recently [20, 21], and will no doubt continue to develop [22].

Note 2. Drug Scaffolds: Pharmaceuticals that have already been used as part of MT-inhibiting photopharmaceuticals include taxol [14] and epothilone [13] for MT-stabilising photopharmaceuticals AzTax and STEpo; and *colchicinoids* (analogues of colchicine-binding-site drugs like colchicine, colcemid, nocodazole or combretastatin) have been used in MT-destabilising photopharmaceuticals e.g. PST, SBTub and PHTub (**Fig 5**) [2, 7, 9, 15, 23, 24].

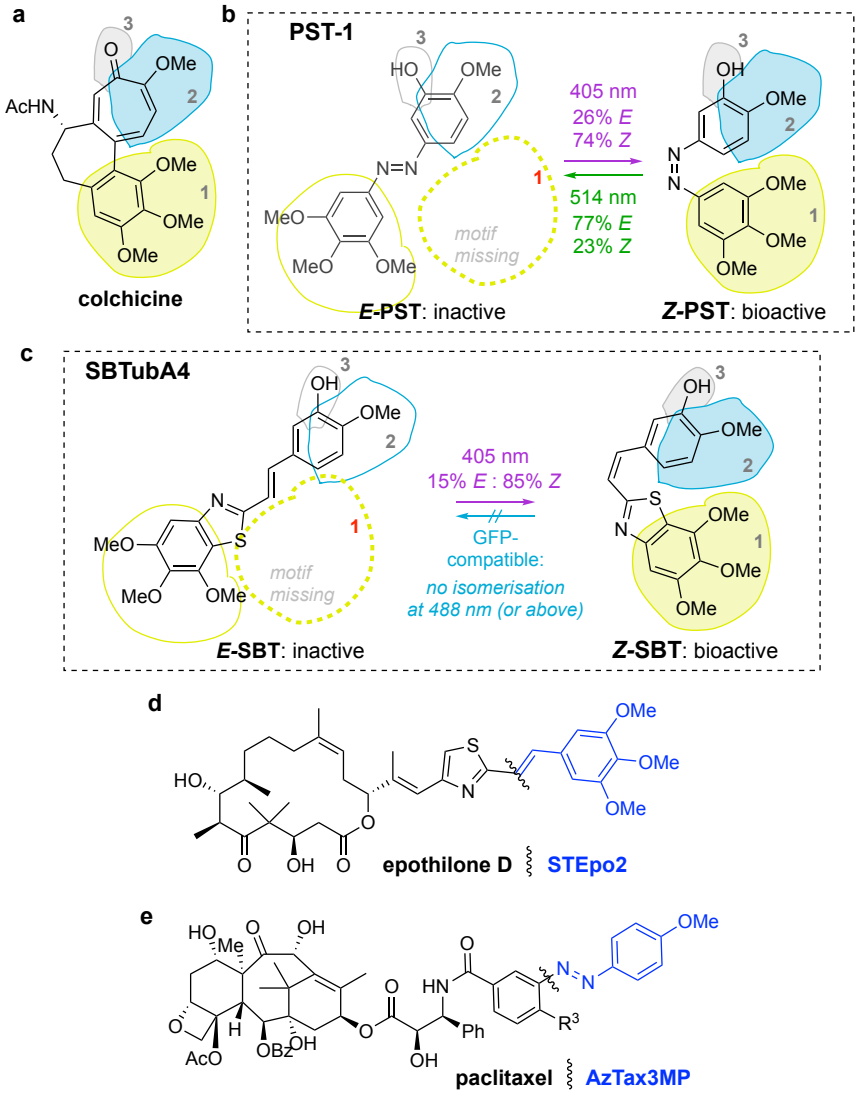

Figure 5: Structural basis for the development of MT-inhibiting photopharmaceuticals, and two examples of their shape change upon *E*↔*Z* photoisomerisation. (a) colchicine's binding to tubulin relies on a pair of structural motifs (labelled **1** and **2**) being held at a fixed distance and orientation; motif **3** is optional but beneficial. **(b-c)** PST and SBTub are colchicinoid photopharmaceuticals

where the photoswitchable bond is inside the overall binding motif. *E*-PST and *E*-SBTub isomers place the **1** motif at the wrong distance and orientation so do not bind tubulin, whereas the *Z*-PST and *Z*-SBTub photoisomers do display the motif pair at the correct distance and orientation, and so have potent tubulin binding. **(d-e)** STEpo and AzTax are created by extending the photoswitchable bond from the periphery of the overall binding motif. They therefore feature residual binding activity in their less active isomers, because the overall structural change upon photoswitching is not large.

Note 3. Mechanism of Action of Photopharmaceuticals: Before use, it should be checked critically whether a photopharmaceutical has been validated as preserving the mechanism of action of their parent drug as their major cellular mechanism, since this cannot just be assumed. Since photopharmaceuticals are typically much less potent than their parent drugs, it has often been seen that using a photopharmaceutical at the (higher) concentrations required to elicit similar on-target responses as the parent drug, results in the photopharmaceutical displaying multiple undesired off-target effects which may even become the primary mechanism of action. We recommend that biologists/microscopists evaluate the available data, and check the major mechanisms of action themselves, before proceeding to research uses. It may be helpful for relatively untested compounds to first consult a colleague in photoswitch chemistry, since some typical off-target effects can be anticipated from considering compound structures.

We suggest to complement all assays with a *permutation control*: that is, using a control compound with a nearly-identical photoswitch and parent drug structure, but where a pair of key substituents have been swapped in a way that prevents *on-target* drug activity. If the permutation control is inactive under dark and illuminated conditions up to ca. 5-fold higher concentrations, then the effects of the photopharmaceutical are likely to be target-specific. Most photopharmaceuticals should have permutation controls available. Typical off-target effects include (**a. Aggregation**): hydrophobic effects, such as surfactant-like effects, protein aggregation, membrane disruption, and compound aggregate formation [25] which are rarely been tested in photopharmaceuticals; (**b. Phototoxicity**): general phototoxicity, and specific CALI (targeted photobleaching of markers on the photopharmaceutical's target). This affects some classes of photoswitch (spiropyran, HTI) more than others (ST/SBT); and nearly all azobenzenes seem free of phototoxicity. Illumination protocols can also cause phototoxicity via endogenous chromophores. A well-chosen permutation control, and careful attention to phototoxicity and solubility, can identify or avoid problems regarding the mechanism of action. Examples for permutation controls include: SBTub4 as a permutation control for SBTub3; PST-28 as an acceptable permutation control for PST-1[P]/PST-2[S]; etc.

Note 4. Photoswitch Scaffolds: Common photoswitches in use in cell biology include derivatives of azobenzene (azo), styrylbenzothiazole (SBT), styrylthiazole (ST), and hemithioindigo (HTI). (**a. Photoisomerisation**) Azo and HTI are *bidirectional photoswitches*: populations can be substantially photoisomerised in both the E→Z and Z→E directions, although only azo is routinely used with bidirectional photoswitching in cells. In a cell biology setting, SBT and ST are effectively *unidirectional photoswitches* that can only be photoisomerised in the E→Z direction. (**b. Metabolism**) Some azo compounds are metabolised in cells, and metabolism of the *Z*-azo (probable timescale of minutes to hours) is ca. 100-fold faster than of the *E*; many *Z*-azo compounds are even degraded by phsiological levels of GSH. SBT is metabolically robust (and the similar ST is likely to be so as well); HTI have not been extensively evaluated but are at least robust to physiological GSH. (**c. Diffusion**) All photopharmaceuticals can rely on diffusional exchange with the medium to continuously return photoisomerised regions to similar Ψ as is present in the medium. Diffusion occurs at different rates for different classes of drug and of photoswitch. For example, small colchicinoid SBTs seem to diffuse much faster (in 2D cell culture, timescale under 1 min) than small colchicinoid HTIs [7, 15]; while larger taxol- or epothilone-based photoswitches seem to diffuse slowly (in 2D cell culture, timescale of minutes) although this apparent slowness might also be due to their stabilising mechanism.

Note 5. Non-ideal Photoswitches. One problem is the residual activity of the less active state: since the photoswitch itself forms only a part of the whole photopharmaceutical structure (see **Fig 5d-e**), it is rare that isomerising the photoswitch can completely abrogate *all* binding of the entire photopharmaceutical, so there is almost always background activity (which may be high; see **1.3**). A very rare exception, are certain colchicine-site MT photopharmaceuticals that have almost zero residual tubulin-binding activity in their less active *E* isomers (PST-1, PST-2, SBTub3, SBTub2M, SBTubA4, and their prodrugs PST-1P, PST-2S, SBTub3P, and SBTubA4P; see **Fig 5a-c**); this

enables their use *in vivo*. Typically, a "good" photopharmaceutical would have a ratio of bioactivity between the more and the less active isomers of ≥10 and such a ratio is probably needed for work in 3D models; an "acceptable" photopharmaceutical would have ratio ≥5 and could be used in 2D cell culture (this includes STEpo and AzTax reagents for MTs); the lower limit for cell-free use is probably around 3-fold. Many other practical limitations, such as the incomplete photoswitching discussed in section **1**, affect all photopharmaceuticals and these limitations prevent them (even the cited colchicine-site photopharmaceuticals) from photopatterning on an "ideal off/on" model.

Note 6. Dark conditions and storage. "Dark conditions" implies, minimal exposure to any light wavelengths that could isomerise the photopharmaceutical, i.e. blocking ambient light below 550 nm. For best results, handle compounds where possible in a darkroom under red light (eg. using colored lamp set to red, lamp with red light bulb as used in photo development, or red LEDs). Ambient white, ultraviolet and violet light can already activate photopharmaceuticals: so ensure these are not present. Once assays are working reliably it is usually possible to reduce precautions to a convenient level and confirm that assays are unaffected.

Stocks: should always be stored wrapped in aluminium foil. For storing DMSO stocks we prefer brown 1 mL glass vials (brown glass absorbs nearly all light below 560 nm) with a PTFE-rubber septum under the cap that makes a tight seal. These are available in packs of 100 or 2000 from chemical suppliers, for example, for analytical HPLC injections. Alternatively, opaque Eppis can be used (these can be white or brown, rather than black - it makes it easier to see the remaining stock solution) and stored in the fridge.

Storage: None of the MT reagents we present requires freezing. In general, small molecule reagents *either as solids or in pure DMSO solution* are remarkably stable. They are (a) completely robust to unlimited freeze-thaw cycles, and (b) stable in the fridge for at least 2 years (longer not tested); though stocks of PSTs were tested stable in DMSO at room temperature in the dark for at least 5 years. In case a stock is ever frozen, the septum ensures good closure contact that stops leakage during freeze-thaw cycles. Unless the cap has a septum, the vial should not be frozen. We prefer not to store DMSO stocks in Eppis at room temperature since it is possible that components leach out of the plastic over time. Eppis with DMSO stocks should also not be frozen unless tightly wound with parafilm, or else they will leak after a few freeze-thaw cycles.

Note 7. Spontaneous Relaxation. For the reagents and applications we describe, this relaxation process is slower than most assay timescales so we will ignore relaxation *in the biological sample*. However, if a stock in DMSO has been accidentally exposed to light, it is usually possible to relax the stock completely back to the all-*E* dark state just by putting the stock (tightly capped, wrapped in aluminium foil) into a drying oven held at 50-70°C overnight: an important advantage compared to photouncaging.

Note 8. Applying Too Much Light. This is usually futile because in our experience these photoequilibrium states or "PSSs" have usually been reached. On most setups, PSS should be reached after a comparable photon flux as would be needed for 2-5 imaging frames (see **Note 17**); applying light beyond what is needed will only bleach the imaging markers and cause other photodamage. If no effect is seen, usually not enough drug has reached the target cells and higher concentrations should be used (see assay development guide in **Methods, 3.1-3.2**).

Note 9. Choosing best practical wavelengths. Often the available wavelengths are fixed microscopy laser lines e.g. 355, 405, 440, 488, 514, 561. Tunable or "white light" lasers (typically only tunable from >480 nm) can be helpful to deliver illumination at exactly one or both of the reagent's optimal photoconversion wavelength/s. The most flexible light sources are monochromators giving any wavelength >340 nm however these do not have spatial precision. Photoswitching is technically possible by two-photon excitation but in practice this is not used because the tiny activation volumes do not isomerise enough compound to deliver biological effects (see also **1.3**).

Since all chemical photoswitches respond broadly to illumination, if a photopharmaceutical is quoted as best responding to 423 nm it is almost certainly fine to isomerise it at any wavelength from 400-450 nm, or even more broadly. Emissive LEDs also have a large spectral bandwidth with typical full width at half maximum (FWHM) intensity of 30 nm, sometimes even up to 100 nm for green LEDs. Caution: some LEDs use phosphors to down-convert light; these will have complex emission spectra (declared on their datasheets) and they should be avoided.

Adjusting tunable or monochromatised light sources can improve the isomerisation Ψ: e.g. for PST reagents, setting λ_2 = 535 nm would give 14:86 *Z:E*. Whether such an improvement is significant or not depends on the assay setting and on which isomer is the more bioactive one (see below: in this case, it would be significant).

Note 10. Photopharmaceuticals are also isomerised by imaging. Ψ(λ) will show what the influence of different wavelengths such as focusing light, or imaging wavelengths, would be. Chemical photoswitches are all strongly light-absorbing species; all photopharmaceuticals will isomerise at any wavelength shorter than its "technical best" isomerisation wavelengths, most will also isomerise up to ca. > 100 nm longer wavelengths than their "technical best". Both must be avoided if possible. We find that photoswitching *under focused laser intensities on the microscope* becomes negligible only when the photopharmaceutical's absorption coefficients are less than 1/1000 of their maximum values, which is only visible on a logarithmic scale plot. In the example of **Fig 1c**, this limit is still not reached by 560 nm, explaining the experimentally verified isomerisation by PST-1 under 561 nm imaging.

Note 11. Establishing Cellular Concentrations Reproducibly: Even at equilibrium, the intracellular concentration of a photoswitch is likely to be different from the bath concentration, and the relationship can be complex (estimates range up to 100-fold higher intracellular concentration [26]). Until equilibrium is reached, while a compound is still diffusing into a cell (or, during washout, is diffusing out of the cell), the current concentration cannot be predicted. Most photopharmaceuticals are entirely nonfluorescent; and fluorescent conjugates of photopharmaceuticals tend to be pharmacologically inactive while also diffusing at vastly different rates from the photopharmaceutical itself; so those approaches cannot be used for tracking concentrations.

We have found that the best way to achieve reproducible results is to firstly ensure reagent solubility by appropriate dilution procedure and final cosolvent content (see **Note 12**), and using as low final concentrations as are feasible to see effects (see **Methods 3.2**). Then, ensure that the applied concentration has equilibrated to the intracellular environment before proceeding. This can be a trial and error step. Equilibration rate depends on the rate of a photopharmaceutical diffusing into cells; and this rate depends on the experimental setup as well as the applied extracellular concentration. If photoswitching is tried long before equilibration, then only a small fraction of the potential photopharmaceutical will have reached the cells of interest, so effects can be weaker or absent, and/or one can be tempted to increase concentrations drastically which usually brings other problems. (**a: 2D**) Reaching sufficient cellular concentrations should take only seconds in 2D cell culture settings where cells are grown on uncoated glass or plastic surface and are maintained under liquid media; we suspect that full equilibration typically takes about 2 minutes. Note that some compounds (e.g. SBTubA4P, PST-1P, PST-2S, SBTub3P) are prodrugs; these can take longer to equilibrate since they need an enzymatic cleavage step to release the photopharmaceutical drug, and they may have slower transmembrane diffusion compared to the drug (in all other respects the prodrug formulation is usually a great advantage, as they usually have much better solubility and handling, which can otherwise limit reproducibility). (**b: Matrix**) Any extracellular sinks that can adsorb or concentrate the typically hydrophobic photopharmaceutical, can necessitate using higher concentrations to ensure that enough compound reaches the test system despite these losses, and longer equilibration times (it is more practical to use higher concentrations as tolerated by the compound solubility and cosolvent limits). In the presence of sinks, we estimate equilibration can take 10 - 60 min. Typical sinks include (i) collagen/agarose matrixes whose fibres may adsorb the photopharmaceutical (workaround: matrices can be cast in solutions containing the photopharmaceutical to reduce losses), (ii) yolk sacs in embryos, which contain much lipid and protein (no workaround known; use higher concentrations). (**c: 3D**) Any barrier between the target cell and the applied medium will slow down the rate of equilibration and can necessitate longer loading times and/or higher applied concentrations, since they stop convective flows from bringing fresh compound into contact with the test system and present direct barriers to cellular entry from the medium. Typical barriers include (i) a matrix that covers the cells (workaround: matrices can be cast in solutions containing the photopharmaceutical), (ii) a chorion surrounding an egg or embryo or cell (workarounds: eggs can be permeabilised by RNAi, or even microinjected with highly concentrated stocks of buffer-soluble compounds if needed; embryos can be dechorionated; neuronal sheaths can be nicked or demyelinated), (iii) multiple intervening cell

layers between the surface-exposed cells and the target cell that will be photocontrolled in a 3D setting (no workaround known). With barriers in place, equilibration may require above 60 min, but this is highly dependent on the experimental setting.

Note 12. Solubility, Dilutions, Checking: Typically, prepare 100X drug dilutions by adding reagents to a tube in this order: DMSO, drug from pure-DMSO stock, media. If compounds that require DMSO for solubility are mixed into cell media with insufficient DMSO they will precipitate from solution and this is irreversible even DMSO is added afterwards. Ensure DMSO used for stocks is good quality (e.g. grade for compound library screening) and is handled cleanly: contaminated or impure DMSO is more toxic to cells. "Dry" DMSO is much more expensive, and is likely to contain more cell-damaging impurities, as compared to compound screening grade. We suggest opening a new DMSO bottle or aliquot every 6 months, as a cheap precaution to prevent contamination. It seems that compound screening grade (usually 1 L bottles) is just as suitable, but also much cheaper than, molecular biology grade (usually 10-100 mL bottles).

Isomerising a DMSO stock sets the *Z:E* ratio in the stock. As long as one isomer does not precipitate out selectively after dilution with cell medium (see below), that same *Z:E* ratio is preserved in all subsequent dilutions. Therefore a "lit stock" can be used for benchmarking expected cellular effects.

Differential solubility of *E* and *Z* isomers: For many azo and ST/SBT reagents, the *Z* isomer is many fold more soluble than the *E* (> 5-fold). Since the dark state is usually the all-*E*, it can happen in that in one assay setup, a compound is photoswitched, and its increased solubility causes biological effects to be seen; whereas in a different assay where the readout is more sensitive or the solubility is reliably maintained, no *E:Z* bioactivity difference would have been seen since the less soluble isomer was already in solution to start with. Isomer-selective precipitation (upon dilution of DMSO stocks with cell medium) can also happen during benchmarking of lit vs dark stocks; it is usually the *E* isomer that precipitates; we typically observe it with above 25 μM of compound and only 1% DMSO, hence the rule of thumb that 1% DMSO should be present per 10 μM of reagent for reliable results.

The best way to check whether precipitation has taken place either in a 100X stock or in final solution is to take absorption spectra (340-600 nm). Typically a concentration of 15 μM or more is needed. Compare to the absorption spectra in a dummy run where instead of media, DMSO is used (all-DMSO mock). If the absorption intensities are similar to within 20%, no precipitation has taken place. If precipitation has taken place, the media-containing setup will have >50% lower absorption intensity and probably the "absorption" will be tail off slowly, but still be significant past 550 nm. Absorption past 550 nm is another proof of precipitation as it indicates fine particulate precipitate in the solution that is scattering light. Expect the *E* isomer to be worse soluble than the *Z*. By regularly measuring absorptions before and after assays, if anything goes wrong, differential solubilities can be confirmed or eliminated as a source of error. Wherever possible, use maximal biologically tolerated cosolvent and maintain all plates warm, to prevent risks of precipitation, until the assay results have been benchmarked.

Note 13. uses of SBTubs and PSTs *in* vivo. SBTubs: For cell-precise and temporally reversible control over microtubule dynamics, in 2D cell culture SBTubs display diffusional reversibility with halftime ca. 20 s, *in vivo* in zebrafish this lengthens to ca. 5-10 min presumably due to hindered access to the medium. SBTubs have been used also for temporally precise blockage of development in frog; and cell-precise targeting of anti-migration and anti-mitotic effects over 24 hours in organoid cultures in a matrix [9]. SBTubs have shown some ability to apply subcellularly-localised effects in large neurons [7]. PSTs have been used to reversibly block/unblock mitosis in worm embryo [2]; manipulate MT dynamics and organising structure in the mouse embryo [27, 28], tissue mechanics in the developing fly [29], redirect migrating cells in culture [30], locally affect neurons [8], and block migrating neurons *in vivo* in zebrafish [31].

Note 14. Hemithioindigo (HTI)-based colchicinoids. HTIs have the unique feature of permitting all-visible-light switching with 440 or 488 // 514 nm laser lines, although their promise for MT photopharmaceuticals is not yet realised. HTI-based colchicinoids PHTubs (e.g. PHTub--7) have been applied in short-term live cell MT photocontrol assays, although HOTubs and HITubs were only tested in long-term applications. Intriguingly, these compounds

seem to show much slower diffusion than comparable small molecules.[15, 23, 24] Their solubility is moderate at best; and they display at best only ca. 10-fold ratio of bioactivity between the isomers, whereas PSTs and SBTubs can have ratios ≥200 making the latter much simpler to use.

Note 15. EB markers. Fluorescently-labelled fusions of the MT end binding protein EB1 or EB3 can be imaged to directly monitor MT polymerisation dynamics, revealing the tips of polymerising MT plus ends in cells as hundreds of dynamic "comets" that cascade through the cell at significant velocities (tdTomato excitation at 561 nm).[32] EB1 and EB3 are functionally equivalent as fluorescent markers. EB-GFP is a good option for GFP orthogonal compounds such as SBTub and STEpo; EB-tdTomato is a good option for azobenzene-based (non-GFP-orthogonal) compounds such as PST and AzTax.

Note 16. ComDet. ComDet plugin for Image J and instructions can be found on: https://github.com/eka-trukha/ComDet

Note 17. Laser power. On a spinning-disk confocal, for imaging EB3-tdTomato at 561 nm, we would typically use 0.04 $\mu W/\mu m^2$ ca. 300 ms per field of view frame. For photoactivation we would typically start with applying 405 nm light at 0.03 $\mu W/\mu m^2$ power in the field of view (again with each "frame" of photoactivation lasting 300 ms, and typically 5 activation frames as a starting value for titrating effects). In our experience, SBTubs respond so efficiently that in 2D cell culture only one photoactivation frame is needed; other compounds with lower-efficiency response to 405 nm will require correspondingly more frames (though in our experience usually 5 is enough, and more than 20 indicates a problem with the assay setup). This highlights that photoswitching can require similar or less light than a few typical imaging frames.

Note 18. Internal controls. To best prepare for cell-localised photoswitching, select a field of view with two well-expressing cells, and use one as an internal control for the illuminations applied to the other, during a similar cycle.

5. References

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