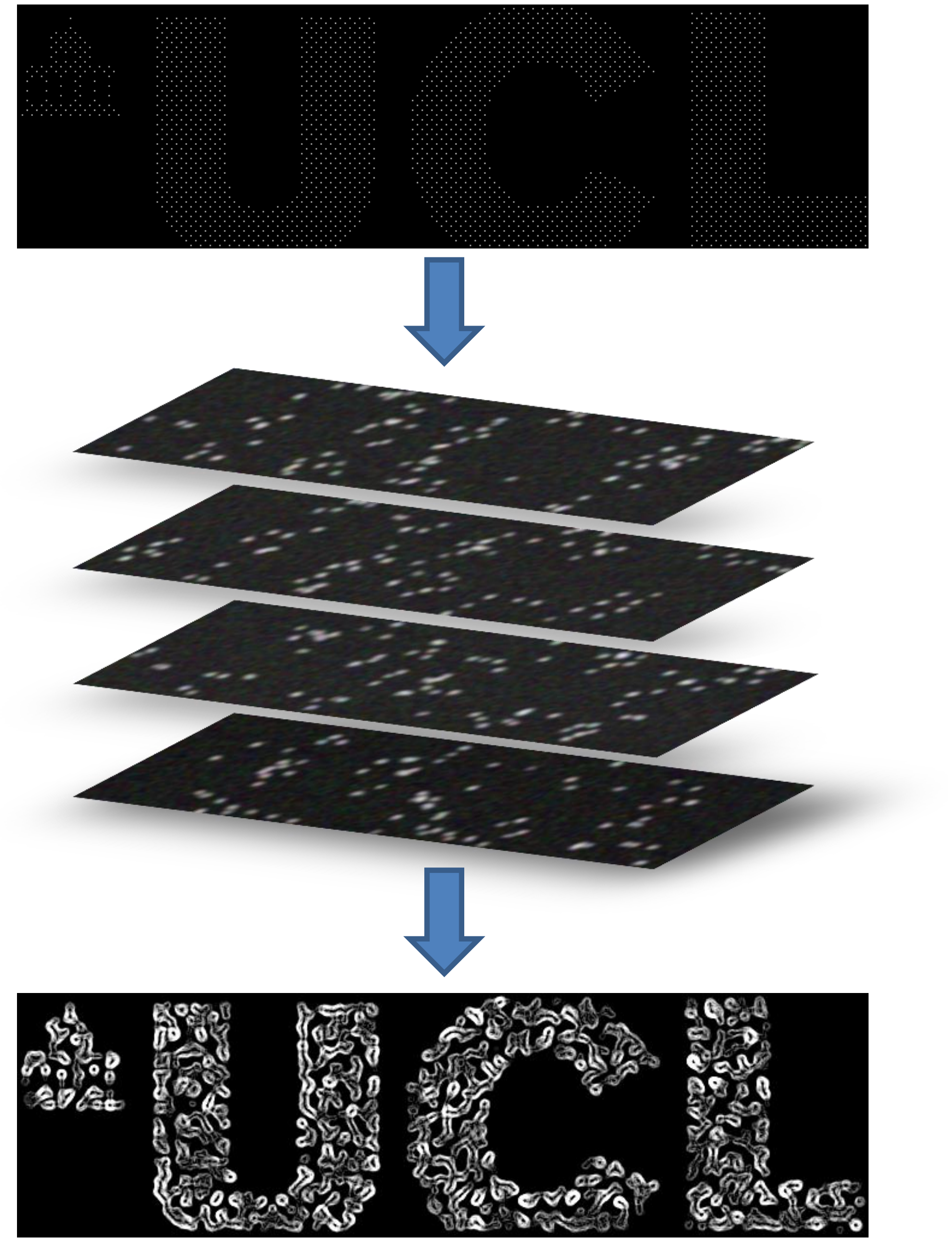
OPTICAL SUPER-RESOLUTION IMAGING: APPROPRIATE DNA LABELS AND IMAGING CONDITIONS



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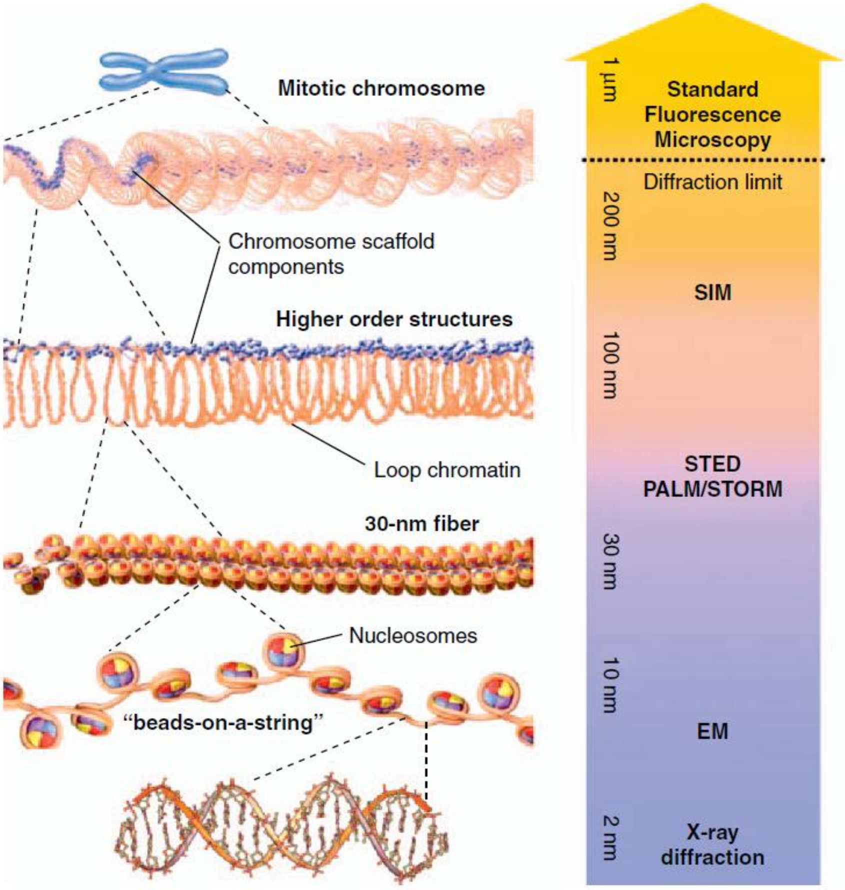
1. INTRODUCTION



Current Opinion in Chemical Biology

Figure 1 : The structure of the chromosome and current microscopy techniques we have to study it; reproduced from Flors and Earnshaw[15]

Recent advances in microscopy have allowed us to see organisms and their structures on a scale many thousands of times smaller than van Leeuwenhoek could ever have envisaged when he used his home-made instruments to study biological samples in the 17th and 18th centuries. Van Leeuwenhoek's finest achievements include the discovery of bacteria and the vacuoles of cells, both of which lie on the micrometre scale. Modern light microscopes allow us to see cells in great detail, although classical light microscopy techniques are limited to resolutions of around 200nm, the diffraction limit of light defined by Abbe.[1] With electron microscopy, resolutions on the picometre scale have been achieved using high-performance instruments; more standard scanning and tunnelling electron microscopes can resolve features on the nanometre scale. Unfortunately, some of the most interesting biological features we wish to study lie tantalisingly out of reach, in between the ranges visible by standard light microscopy and electron microscopy (1-100nm) (figure 1). In the last two decades, a plethora of new techniques has been introduced, allowing features to be resolved on ever-smaller scales using optical microscopy. It is through such improvements that new structural data on a variety of biological systems have been elucidated.

Of all the structures in the cell, the chromosome is arguably one of the most fascinating. The chromosome represents a highly efficient packing system of DNA within the cell – each chromosome comprises a single strand of DNA which wraps around protein units to form fibres, which in turn wrap around more scaffold proteins. In this manner, a three-metre strand of DNA is rather remarkably condensed into a structure on the micron scale. The structure of the condensed chromosome and the mechanisms by which it reaches this state are only partially understood, despite advances in microscopy in recent years. A better understanding of the structure and replication of chromosomes would be beneficial to those researching genetic conditions: many genetic illnesses occur as a result of a chromosome being replicated incorrectly.

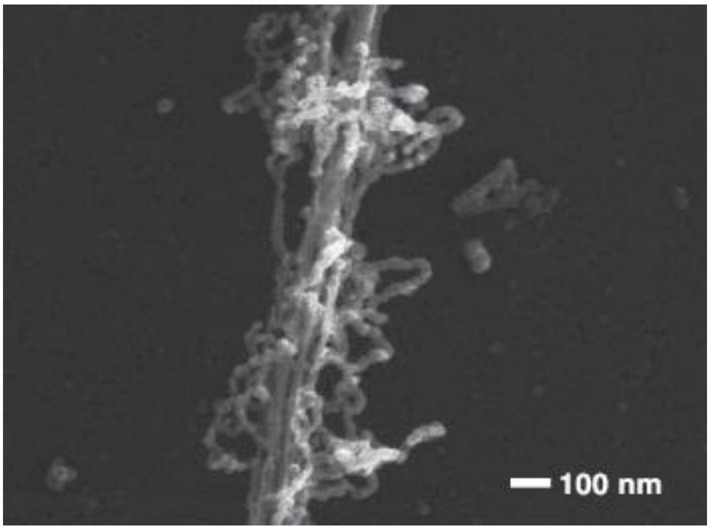


Figure 2 : SEM image of barley metaphase chromosomes. Decondensed DNA wrapped around the protein scaffold is clearly visible; reproduced from Wanner et al.[3]

The most recent significant advances in understanding the structure of chromosomes were those made by Wanner's group[2,3] using electron microscopy; the group was able to obtain images of chromosomes clearly showing the presence of a 30nm fibre within the structure, the existence of which had previously been the subject of some debate. Further advances by Wanner's group include the three-dimensional reconstruction of chromosomes using focussed ion beam (FIB) milling techniques,[4] resulting in the unprecedented characterisation of the interior structure of the chromosome. Despite the impressive nature of these works, the sample preparation techniques employed are not compatible with live samples, severely limiting the study of chromosome replication in real time. The development of new optical super-resolution microscopy techniques therefore presents an unrivalled opportunity to study the structure of the chromosome below the diffraction limit. Live-cell[5], multicolour[6] and 3D[7] optical super-resolution microscopy methods have already been demonstrated, and it is conceivable that by exploiting the high resolutions and in vivo capabilities of such techniques that the underlying mechanisms of chromosome replication can be ascertained. This review therefore provides an introduction to popular super-resolution techniques, the photophysical characteristics of fluorophores used and the imaging conditions used to obtain super-resolution images. A final section focusses on the methods available for the labelling of DNA with fluorescent markers.

2. OPTICAL SUPER-RESOLUTION MICROSCOPY

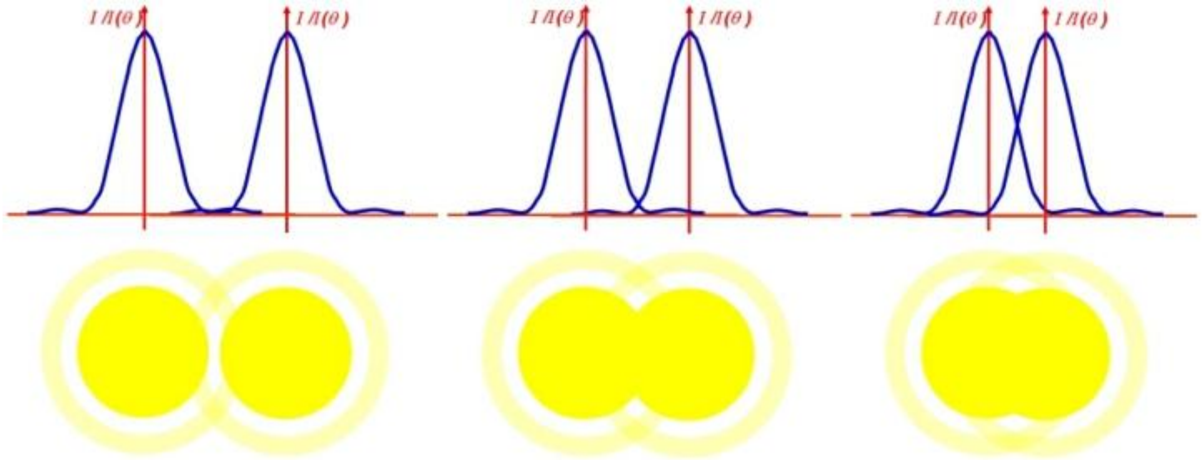


***Figure 3 :* *The Rayleigh criterion - in order for two peaks to be resolved, they must be further apart than Δl, Left – the Airy disks do not overlap; the PSFs are resolvable. Middle – the disks overlap slightly, but the peaks are separated by a distance greater than Δl; Right – the disks overlap and the peaks are separated by a distance smaller than Δl and are not resolvable; reproduced from astronomy.swin.edu*[10]**

Objects on the level of the cell are effectively transparent to the human eye, and so light microscopy methods either require samples to be coloured, or take advantage of differences in phase as light passes through specimens in order to make out their features. Phase contrast methods involve converting the phase shifts in light passing through the sample to variations in brightness in the image,[8] whilst stains increase the contrast of a sample in areas where they are attached. In both cases we can recognise structures within the specimen being studied. An extension of traditional colour staining methods is to use fluorescent dyes, as these have a very large contrast with a dark background when excited using UV (or in some cases visible) light. As with any optical technique, however, the resolution achievable using fluorescence microscopy is limited by the diffraction of light.

The Rayleigh criterion[9] gives a minimum distance for which two light sources can be optically resolved:

$$\Delta l = 0.61\frac{\lambda}{NA},$$

where λ is the wavelength of illuminating light and NA is the numerical aperture of the optical system. The distance Δl represents the separation of the centroids of each marker's point spread function (PSF); the fluorophores are optically resolvable if Δl is greater than the radius of the marker's Airy disk. This is illustrated in figure 3 using Gaussian point spread functions. In the case of biological samples labelled with fluorescent markers, two neighbouring fluorophores active at the same time will not be resolvable using visible light.

Various optical microscopy methods using fluorescent markers have been devised to obtain spatial resolutions below the diffraction limit of light. These have been termed 'super-resolution' microscopy, or sometimes nanoscopy. The most popular techniques fall into two categories, targeted and stochastic super-resolution. Targeted readout techniques reduce the size of the effective fluorescence point spread function (PSF), whilst in stochastic techniques random subsets of fluorophores much further apart than Δl according to the Rayleigh criterion are imaged sequentially.

In targeted readout methods such as Hell's stimulated emission by depletion (STED),[11,12] the sample is scanned with a combined excitation/depletion beam. The depletion beam, which stimulates emission from fluorophores excited by the excitation beam, has the shape of a torus. As both beams are aligned according to the same centre, the effect is that only fluorophores within the inner hole of the torus are illuminated and the effective fluorescence PSF is 'sharpened'. The technique is advantageous in that no image reconstruction is needed, however several scans of a sample may be necessary to record all of the fluorophores. Images with spatial resolutions of ~50nm have been obtained using STED.[13]

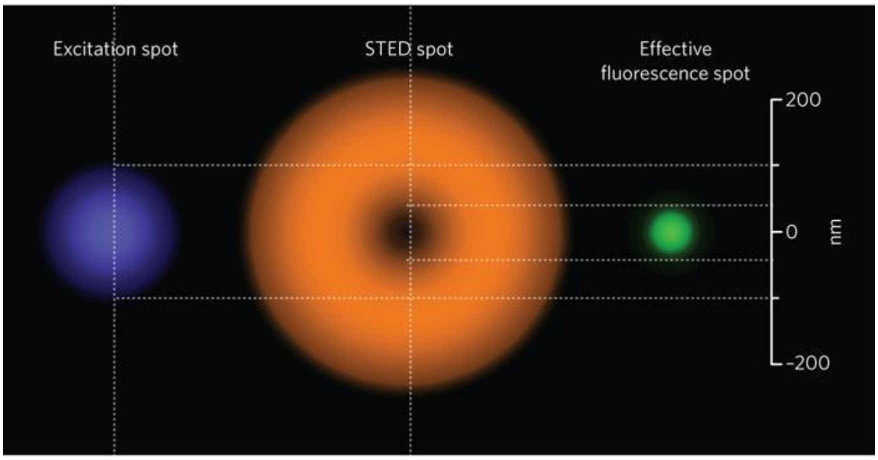


Figure 4 : The STED concept - the normal excitation spot is 'shrunk' using a torus-shaped depletion beam, resulting in a smaller effective excitation spot; reproduced from Abbott[12]

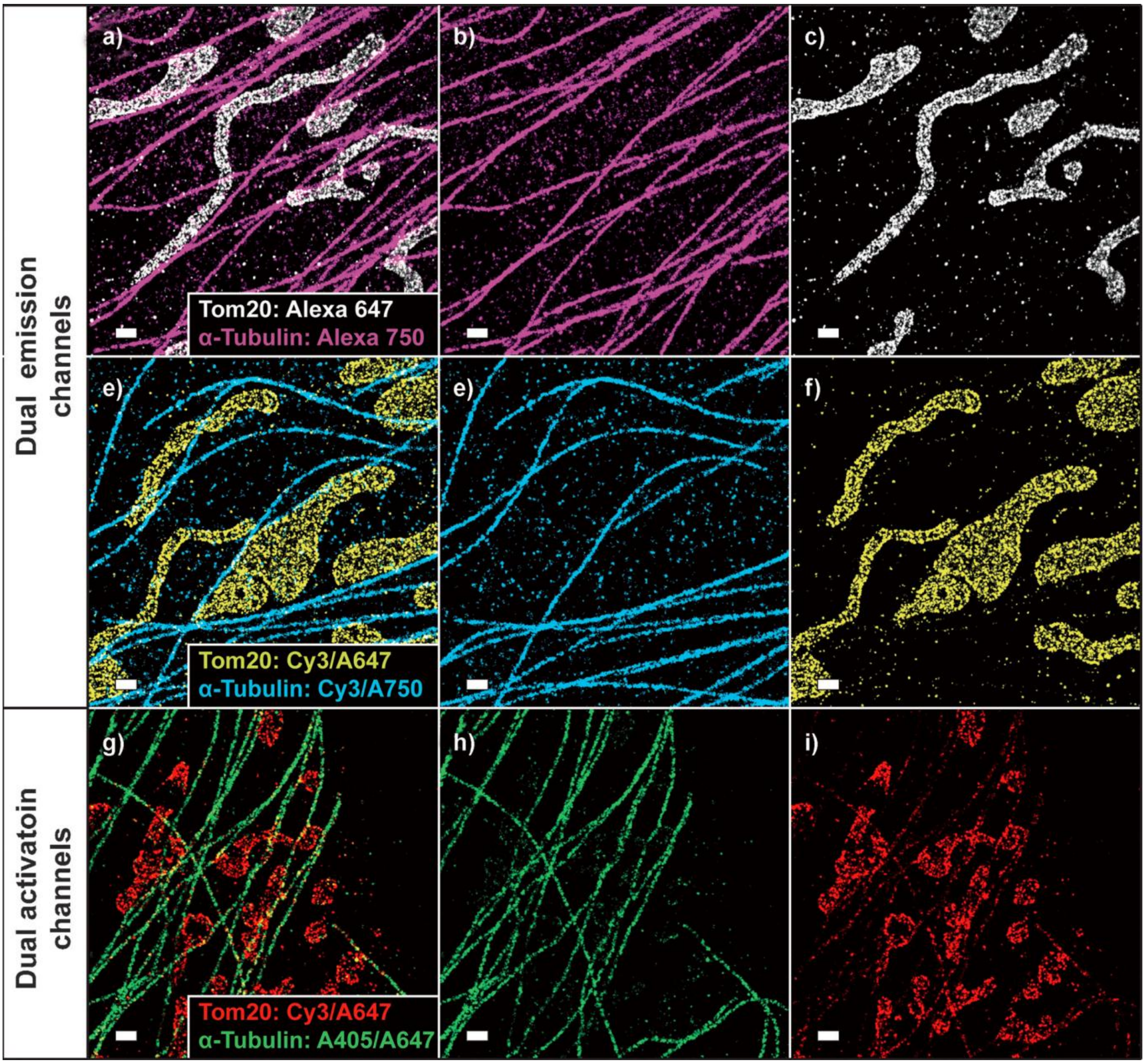


Figure 5: Multicolour super-resolution images reproduced from Zhuang et al.[6] *Dual-emission channel STORM imaging of microtubules and mitochondria in BSC-1 cells immuno-stained with various fluorescent markers. Scale bars 500 nm.*

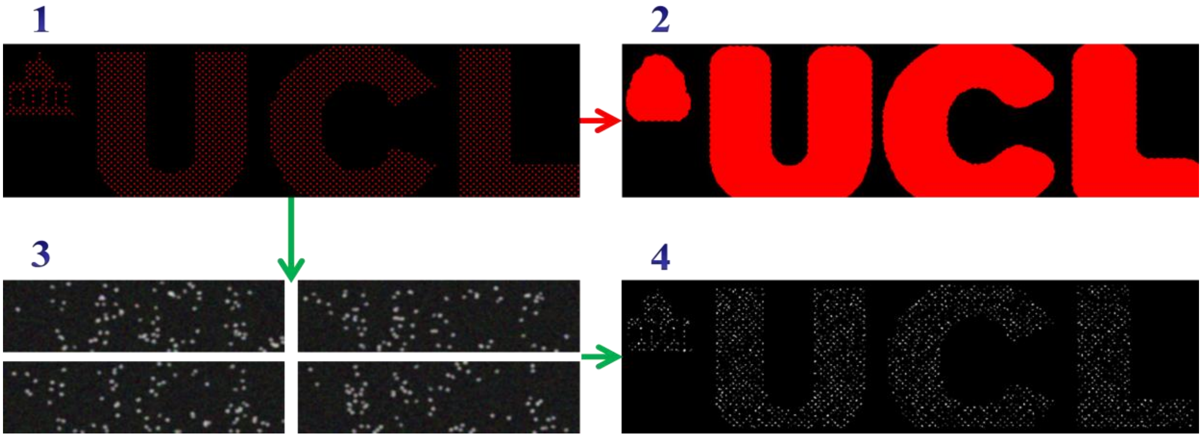


Figure 6: A simulation of a stochastic imaging experiment: the UCL logo is labelled with markers (1) which are not resolvable when excited simultaneously (2). Many subsets of active markers are activated sequentially (3) before a reconstruction is made (4).

Stochastic imaging techniques rely on the activation of a subset of fluorophores[14] spread across the sample with the rest remaining in a dark state, allowing the bright fluorophores to be resolved spatially (figure 6). Probability dictates that if enough subsets of different molecules are ‘switched on’, all of the probes in the sample will have been recorded. Typically, stochastic techniques produce images with spatial resolutions of ~30nm;[14,15] these images are usually obtained using an inverted total-internal-reflection (TIRF) microscope, which minimises the likelihood of background fluorescence from the sample.

Despite the vast number of different stochastic techniques recorded in the literature, it should be noted that all rely upon the ability to switch between different, optically resolvable subsets of fluorophores repeatedly, and that the only major difference is the methods by which the dyes are made to cycle between the on and off states (see section 2.2). The nature of the light and dark states of organic fluorophores can be understood by referring to their photophysical and photochemical properties.

2.1 PHOTOPHYSICS AND PHOTOCHEMISTRY

Fluorophores are designed to be bright, and so before the advent of localisation microscopy, any dimming or blinking of the probes was seen as a nuisance. The realisation that the control of dark states is essential to stochastic imaging methods has led to a concerted effort to study and understand the dark states of these molecules. The following section details the mechanisms of fluorescence and phosphorescence, and the nature of organic fluorophore dark states.

2.1.1 RELAXATION FROM EXCITED STATES

Organic fluorophores contain a highly delocalised electron system which facilitates the promotion of electrons from the ground state to an excited electronic one. Whether an electronic transition will occur or not is governed by a series of selection rules involving the spin multiplicity of the electronic states. A pair of electrons in a given orbital have opposite spin (+½ and -½), and so a closed-shell system has zero spin ($S = s_1 + s_2 = \frac{1}{2} - \frac{1}{2} = 0$) and a spin multiplicity of one, as there are $2S+1$ possible values of the spin angular momentum. Such a state is known as a singlet. If two aligned unpaired electrons are present in the same system, the spin angular momentum will be one and the multiplicity will be three, and the state is called a ‘triplet’.

When an electron is promoted to a higher-lying orbital, it can be either aligned or misaligned with the electron it leaves behind, resulting in the formation of a triplet or singlet state respectively. In conjugated systems, therefore, the ground electronic state is a singlet state, and the most accessible excited electronic state is also a singlet, as singlet-singlet transitions are allowed by the electronic selection rules ($\Delta S = 0$). There are numerous ways for the excited electron to return to the ground state, and these can be categorised as radiative, where relaxation results in the emission of a photon, or non-radiative; the relaxation mechanisms of a fluorophore can be summarised on a Jablonski diagram (figure 7).[16]

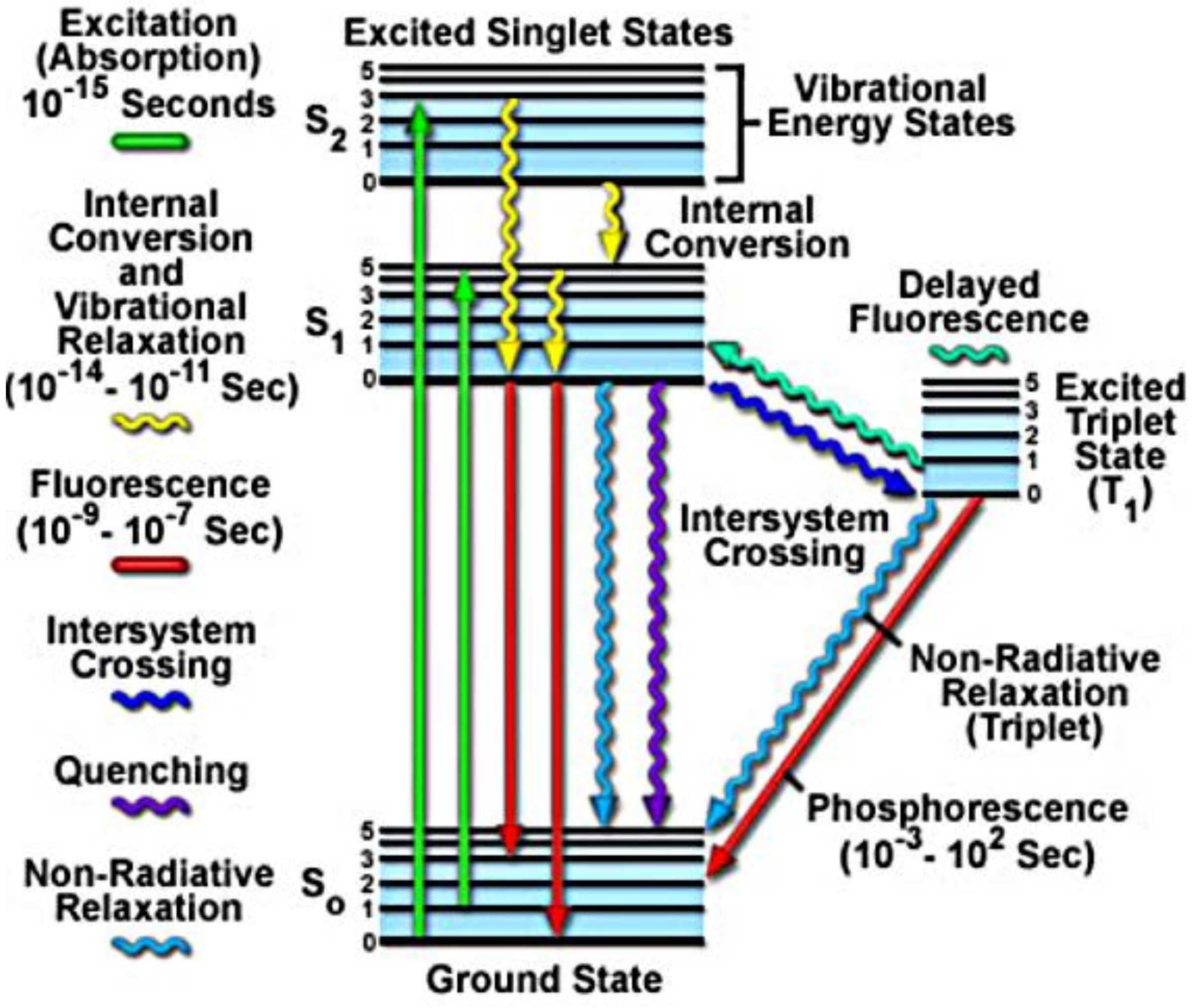


Figure 7 : Jablonski diagram summarising the radiative and non-radiative relaxation pathways of a typical fluorophore; reproduced from olympusmicro.com[17]

The two radiative relaxation mechanisms are characterised by their very different lifetimes. Fluorescence is the name for radiative emission from an excited electronic state of a molecule to a ground state with the same spin and occurs on a nanosecond timescale. Light emission resulting from phosphorescence, on the other hand, can last for periods of up to several hours in extreme cases; notable examples include zinc sulphide and strontium aluminate for use in glow-in-the-dark toys and signs.[18] This phenomenon results from electrons being ‘trapped’ in a triplet state after excitation. The radiative transition to the ground singlet state from the excited triplet state is unfavourable because of the selection rules, however if there are no competing relaxation pathways then the radiative transition will occur, albeit very slowly compared to fluorescence, hence the much longer lifetimes. In some cases, a molecule can spend time in an excited triplet state before crossing back to the singlet state whereupon it fluoresces in a process known as delayed fluorescence.

Non-radiative relaxation mechanisms can be either internal or can involve interactions with other species in the fluorophore’s environment. External conversion tends to involve the dissipation of energy from excited states through collisions with solvent molecules. The main internal pathways are vibrational relaxation, where the energy from the excited electronic state is dissipated through various vibrational states and the fluorophore returns to the ground state, and inter-system crossing, where the fluorophore crosses from the excited singlet state to an exited triplet state; the latter is described by the intersystem crossing (ISC) rate. The ISC mechanism by which triplet states are formed depends on the energy overlap between the vibrational levels of the first excited triplet and singlet states of the fluorophore. The greater the overlap, the more likely it is that the fluorophore will enter the triplet state. The observation that the ISC rate is increased by the presence of heavy atoms such as iodine, selenium or transition metals – the so-called heavy atom effect – is explained by spin-orbit coupling.[19,20,21]

2.1.2 THE FATE OF THE TRIPLET STATE

For an organic fluorophore, an excursion to the triplet state will generally happen once for every thousand singlet-singlet transitions.[22] The fate of molecules once they enter a triplet state is of great interest in the field of photophysics, as it is believed that many of the reactions leading to permanent photobleaching of dyes occur when a molecule is in this state. Phosphorescence is not the main relaxation mechanism from the triplet state in many cases: the probability of the occurrence of radiative transitions in liquid solution is practically zero as relaxation through interaction with external molecules is far more efficient.[23] It is only at very low temperatures or in rigid media that the phosphorescence of cyanine or rhodamine dyes is observed.[23,24]

Chibisov's work of 1977[25] gives an excellent account of the various reactions of the triplet state of cyanine dyes in both rigid media and solutions with varying concentrations of electron donors and acceptors. For a number of different cyanine dyes, Chibisov determined the rate constants for the reactions of their triplet states, into which they had been excited by flash photolysis, and was able to show that cyanines in the triplet state can be both reduced and oxidised in solution to form radical anions and cations respectively. When electron transfer from an external species is involved, external processes are sometimes termed as photooxidation or photoreduction depending on the electron-transfer species.

2.1.3 DARK STATES

The term 'dark state' is generally used to mean any non-fluorescent state of a normally fluorescent molecule. Dark states can be permanent or non-permanent, and most can be exploited to some extent in super-resolution imaging. It is important to differentiate between dark states of a fluorophore which result from the population of electronic states of the fluorophore from which a radiative transition is highly unlikely and those which are the products of reactions with other species to form a structurally different entity.

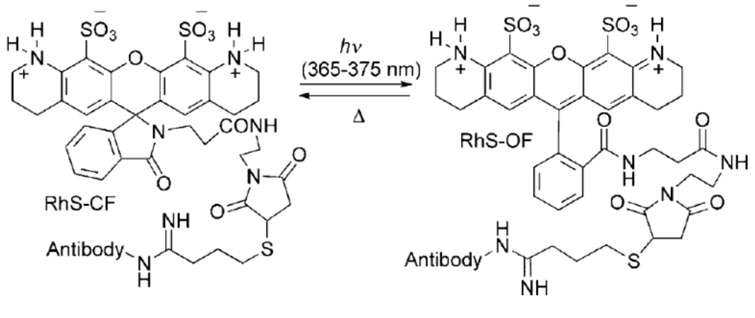


***Figure 8 : Reaction scheme for the reversible photoactivation of a caged rhodamine; reproduced from Belov* et al.**[29]

The triplet state of dyes falls into the first camp, as do the radical ions discussed in the previous section, despite the radicals being formed as the result of a reaction with a redox agent. This is because the structure of the radical is essentially identical to that of its parent dye,[26] but with a lone electron occupying one of the orbitals resulting from a one-electron transfer process. Whilst it has been shown that some radicals show fluorescence,[27,28] it is almost certain that dye radicals do not fluoresce under the same conditions as the parent dye. Radical ions are considered to be non-permanent dark states as they revert to the fluorophore ground state upon oxidation. The triplet state, as discussed above, is unlikely to emit phosphorescence radiation under normal imaging conditions, instead returning to the fluorescent state *via* various pathways, and is therefore considered a non-permanent dark state.

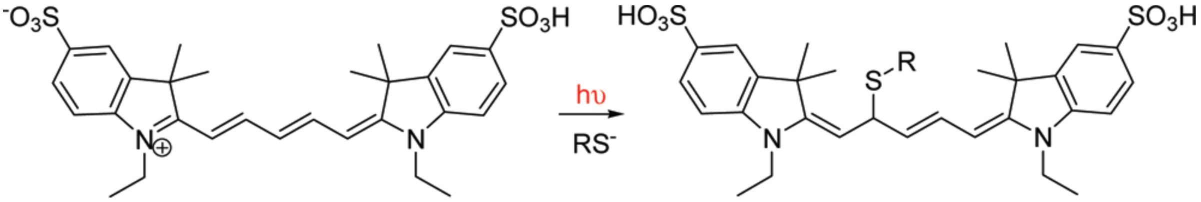


***Figure 9 : The photocatalysed reaction leading to the thiol addict dark state of cyanine dyes; reproduced from Bates* et al.[30]**

Many dyes undergo photocatalysed two-electron reactions to form a different, non-fluorescent species. In many cases these reactions are reversible and can therefore be exploited where robust, controlled switching between a light and dark state is required – indeed, molecules or sets of molecules that display such behaviour are often known as molecular switches. Notable examples include the so-called ‘caged’ rhodamines (figure 8),[29] where a photocatalysed ring-opening reaction extends the conjugated system over the entire molecule, and the reaction of cyanines with thiols to form a non-fluorescent adduct (figure 9).[30] The former reaction is reversed by heating, whilst the second is reversed by the absorption of light of a different wavelength, a mechanism that is used to great effect in STORM imaging (see section 2.2.1).

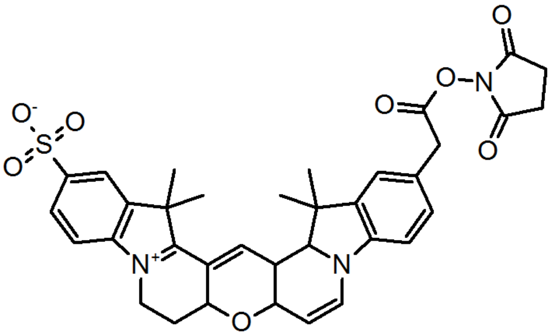


Figure 10 : The Cy3B structure[33]

Cyanines additionally have light and dark diastereoisomers. By virtue of the unsaturated bonds on their polymethine bridge, cyanines display *cis-trans* isomerism, which leads to an unwelcome dimming of fluorescence as the molecule changes from the fluorescent *trans*-isomer to the dark *cis*-form.[31] It is thought that the *cis*-isomer, with a slightly more flexible polymethine chain than the *trans*-isomer, is more prone to non-radiative vibrational relaxation and therefore does not fluoresce.[32] Despite the *cis*-isomer being more stable than the *trans*-isomer for some dyes, in most cases it is not and the isomerism is fully reversible; the *cis*-isomer is therefore a non-permanent dark state. *Cis-trans* isomerisation can, of course, be completely removed by rigidifying the structure of the cyanine, as with the Cy3B dye (figure 10).[33]

2.2 MECHANISMS FOR STOCHASTIC MICROSCOPY

As fluorophore dark states can be separated into those formed following one- and two-electron reactions, it follows that different imaging methods can be similarly divided. We differentiate between photoactivation techniques and ‘blink’ microscopy methods.[34,35] The former involves the activation of a sparse subset of fluorophores – usually of the type of molecular switch described in the previous section – using one wavelength of light and their excitation using another wavelength. Blink microscopy techniques rely solely upon the formation of radical anions from dye triplet states, and are therefore much easier to implement.

2.2.1 PHOTOACTIVATION METHODS

Photoactivation methods rely on the labelling of a sample with fluorescent molecular switches, usually fluorescent proteins or specially-constructed reporter systems. Photoactivated localisation microscopy (PALM)[36] is perhaps the simplest method, as it requires the activation of a sparse subset of protein molecules with one wavelength of light followed by their excitation and photobleaching with another wavelength. This technique, whilst effective, relies on the destruction of the fluorophore and means that each molecule can only be imaged once, reducing the potential accuracy of the technique from the outset. Stochastic optical reconstruction microscopy (STORM)[37] uses a reversible photoactivable probe and is therefore inherently more robust.

The original STORM paper describes the use of an ‘activator-reporter’ pair. This pair comprises two cyanine dyes which absorb at different wavelengths, in this case Cy3 and Cy5, placed in close proximity on the sample; the former dye emits green light and the latter red. As mentioned above, cyanines form a non-fluorescent adduct with thiols, a reaction that is photocatalysed by the light of the dye’s excitation wavelength and reversed by light of a lower wavelength. The existence of this photoswitchable dark state was confirmed by Bates *et al.* in 2010.[30] By employing negative-ion mass spectrometry, the group were able to confirm the presence of a thiol-cyanine adduct after the illumination with red laser light of soluble cyanine dyes in a standard aqueous STORM imaging buffer. Spectra of the

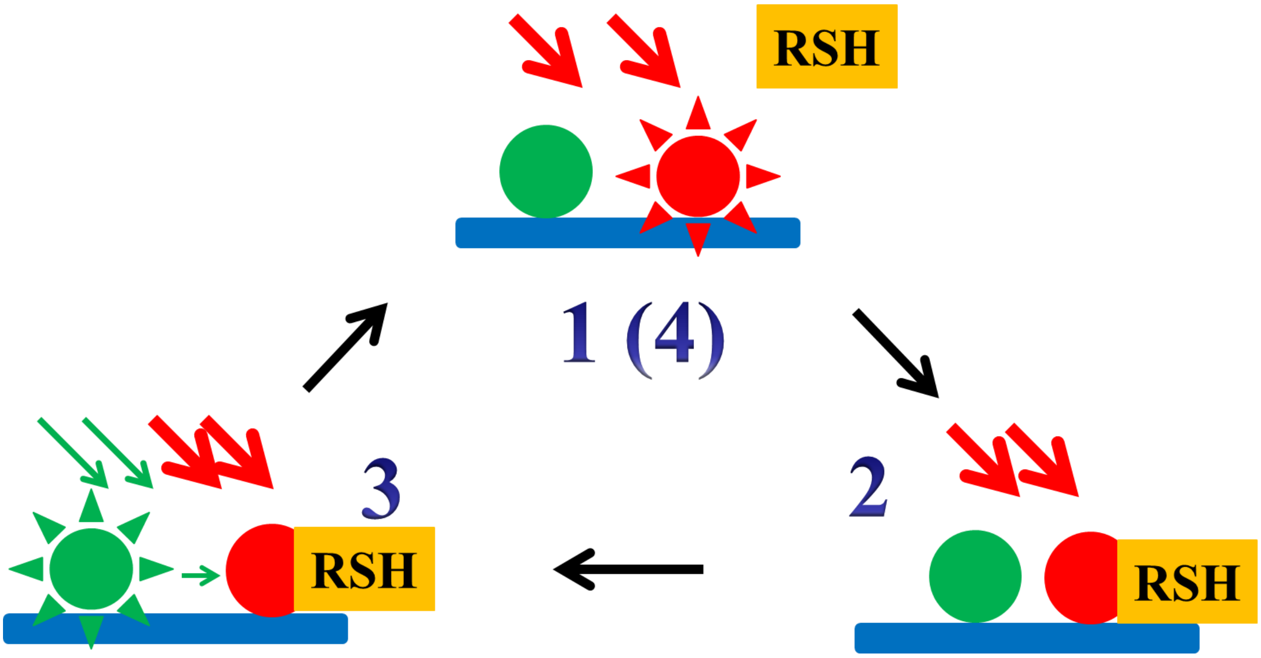


Figure 11: The STORM mechanism
1) Red cyanine marker fluoresces under illumination with red laser light. Inset, Cy5, a popular cyanine marker
2) Excited red dye forms non-fluorescent adduct with thiol
3) Weakly intense green light excites a sparse subset of green markers, the green light emitted is absorbed by the red marker to reverse the adduct formation
4) Sparse subset of red markers is reactivated, fluoresces, and is imaged

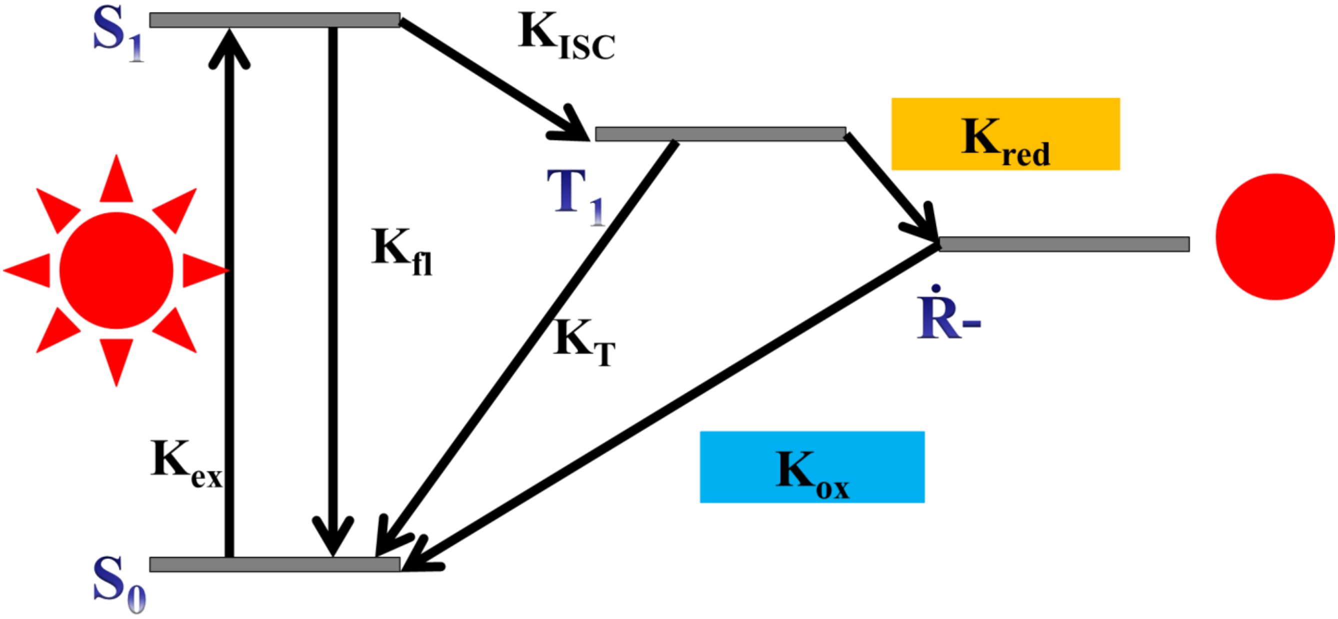


Figure 12 : The Blink microscopy mechanism
1) Fluorophore fluoresces as normal. Every ~1000 excitations it enters an excited triplet state.
2) Oxygen concentration in the solution has been reduced to limit reaction with triplet state and formation of reactive singlet oxygen
3) Triplet reacts instead with reducing agent to form long-lived non-fluorescent radical anion
4) Radical anion is oxidised by remaining oxygen or other oxidant. The fluorescent state is recovered.

solution taken before illumination display peaks corresponding to the mass of the unreacted cyanine, whilst spectra taken after illumination clearly indicate the presence of a species with a mass equivalent to those of the cyanine and a deprotonated thiol combined.

In STORM experiments, the sample, labelled with Cy3-Cy5activator-reporter pairs, is continuously irradiated with red light. Initially, all reporter probes are on, but will react with the thiol in solution to form the dark adduct. When all of the reporters are dark, the sample is irradiated with a weak (~5 W cm^{-2}) pulse of green light, which activates a random subset of Cy3 dyes. The light subsequently emitted by Cy3 is absorbed by its neighbouring Cy5 adduct, reversing the adduct formation and returning Cy5 to the fluorescent state. It should be noted that the Cy3 molecules are not irradiated for long enough to allow a Cy3-thiol adduct to form, so they remain in their 'active' state. As the red laser irradiates the sample throughout the experiment, the newly-reactivated Cy5 dyes fluoresce until they react with the thiol, and all molecules return to the dark state once more. The process is repeated, with an image taken each time the reporter probes are activated. The technique is advantageous as the switching cycle is robust (hundreds of switching cycles) and differential labelling of samples with different colour switching pairs allows for basic colour imaging.

2.2.2 BLINK METHODS

Whilst photoactivation imaging methods are generally reliable, they are slow due to the need to wait for photobleaching or deactivation to occur before exciting a new subset of molecules – a typical PALM experiment can take up to 12 hours to complete. Furthermore, the use of specialised probes or fluorescent proteins is required, limiting the range of samples that can be labelled. Both Heilemann[38] and Hell[39] demonstrated the automatic switching of conventional fluorophores with a single laser excitation wavelength, significantly reducing the complexity of imaging compared to STORM. These techniques were called direct STORM (dSTORM) and ground state depletion followed by individual molecule return (GSDIM) by the respective groups. Collectively, these methods and others derived from them are often labelled as 'blink' microscopy.

The main dark state involved in blink methods is a metastable radical anion formed by a one-electron reaction of the fluorophore excited triplet state and a reducing agent.[40,41,42] An oxygen scavenging system is used to ensure that photobleaching is minimised, as a variety of reactive oxygen species such as superoxide radicals and singlet oxygen are formed from reactions of triplet-state oxygen with the dye triplet states and radicals. A certain amount of oxygen is needed, however, to return the radical anions to the singlet state of the dye through oxidation, so it is fortuitous that the enzymatic oxygen scavenging schemes employed leave a micromolar concentration of oxygen.[22,34] There are several advantages to blink methods, therefore, not least the fact that other families of fluorophores than cyanines can be used, such as rhodamine or oxazine dyes. In addition, the experimental setup is far simpler than the original STORM methods, with only one laser needed. In fact, the use of a thiol and oxygen scavenging system is more of a hangover from the STORM technique, as different reducing agents such as ascorbic acid[5,43]and Trolox,[44] a water-soluble vitamin E analogue, have been used to achieve blinking effects.

2.2.3 REDUCING-OXIDISING SYSTEM (ROXS) FOR BLINK METHODS

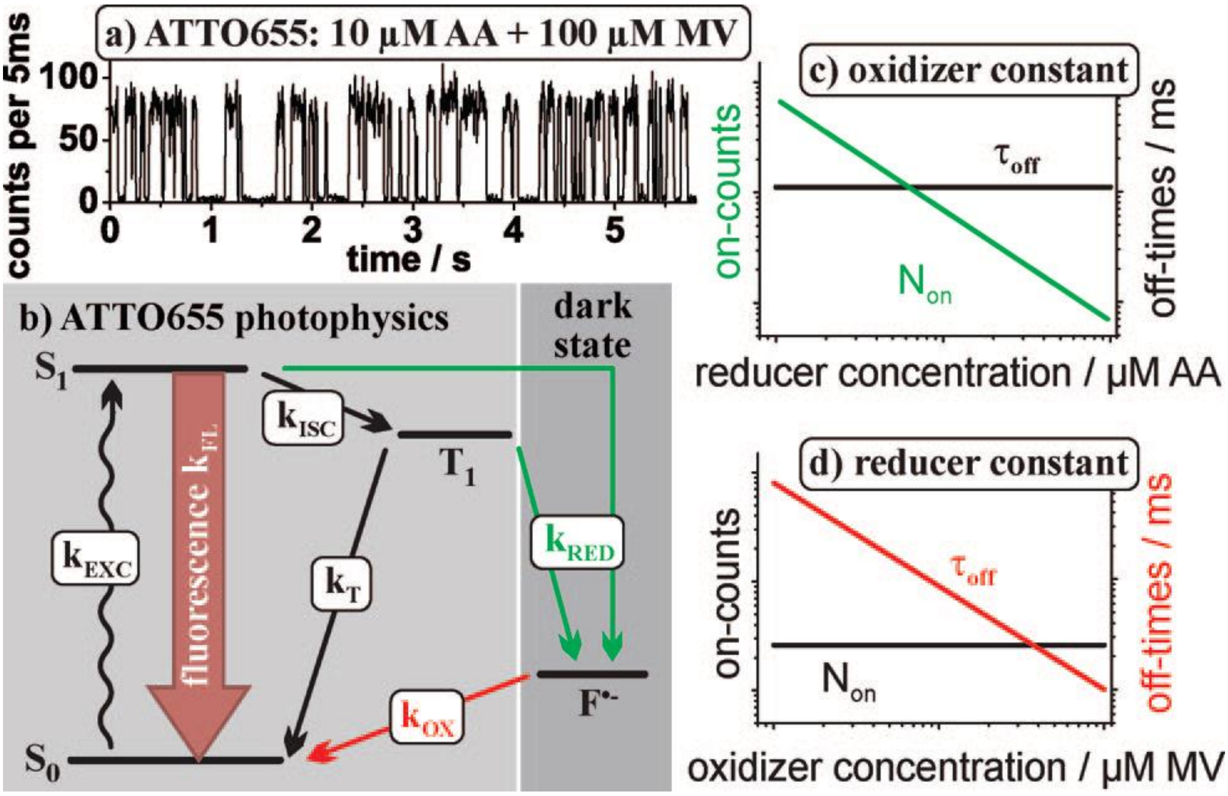


Figure 13 : The ROXS system with updated Jablonski diagram. All states apart from the metastable radical anion are considered to be 'on'. The lifetime of the on state depends on the reductant concentration, the lifetime of the off state on the concentration of the oxidant. Reproduced from Cordes et al.[44]

The origins of the imaging solutions for blink microscopy techniques lie in attempts to prevent photobleaching in fluorophores. Singlet oxygen has been identified as the species responsible for photobleaching[45] and so enzymatic oxygen removal systems are usually employed to reduce the likelihood of formation of the singlet state. It has been observed, however, that the removal of oxygen actually aids the accumulation of metastable triplet states in solution: it is the interaction of ground state triplet oxygen with triplet state fluorophores that returns the fluorophore to the fluorescent state, and therefore a reduced oxygen concentration leads to longer triplet lifetimes.[34] Instead of removing oxygen from imaging media, reducing agents or radical scavengers such as β-mercaptoethanol, cysteamine or ascorbic acid can be added so that the triplet state is depleted more quickly. Oxygen then oxidises the reduced fluorophore, a radical anion, so that it returns to the ground singlet state. There is still the risk that oxygen will react with the triplet state, however its reaction with the radical anion is preferential and so photobleaching is reduced significantly.

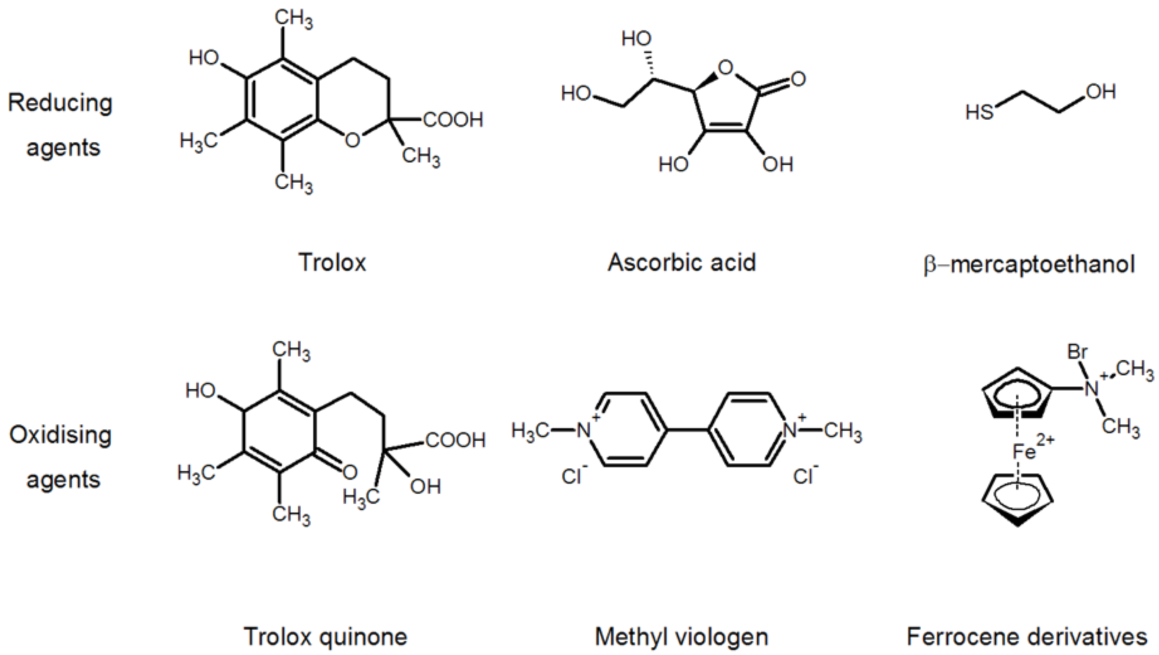


FIGURE 14: *A range of reducing and oxidising agents used in the ROXS system*

Blink microscopy buffer solutions are therefore derived from these attempts to reduce photobleaching, but both oxygen removal and triplet state reduction are employed. This affords good blinking conditions: the triplet state fluorophore is rapidly converted to the radical anion, and the reduced oxygen concentration means that the radical species are longer-lived than usual. In general for dSTORM-type methods, a single molecule will emit fluorescence up to ten times per minute. There are drawbacks to using such imaging conditions, however, such as a lack of control over the blinking rate and the fact that photobleaching is still a possibility. In an attempt to exercise more control over blinking rates, therefore, Heilemann's group introduced the idea of the reducing-oxidising system (ROXS),[46] where oxygen is removed, and both a reducing and an oxidising agent are added to the solution. Suitable oxidants include methyl viologen, ferrocene-based derivatives and nitrobenzoic acid.[47]

Under these conditions, photobleaching by oxygen is minimised, the reducing agent forms the radical anion, which reacts with the oxidising agent to return to the singlet state. The system is summarised in figure 13. It is assumed that in many cases the oxidant is not powerful enough to oxidise the excited triplet state of the fluorophore and that neither the oxidant nor the reductant are powerful enough to react with any singlet state of the fluorophore. Furthermore, the efficiency of both the reduction of the excited triplet state and the non-radiative triplet decay processes means that the triplet state can actually be considered as an 'on' state when an oxidising agent is employed: the triplet state reverts to the fluorescent state much more quickly than the metastable radical.

By using the ROXS system, one can vary the concentrations of the oxidising and reducing agents and thereby control the blinking rate of the fluorophore with much greater precision than with other systems (figure 13).[44,47] It has been shown experimentally by measuring the rate constants for the various processes in the ROXS system that the duration of the 'on' state depends on the reductant concentration and the duration of the 'off' state on the concentration of the oxidant. This has great implications for blink microscopy: fast blinking and therefore short-lived on and off states are required for high temporal resolution in blink microscopy, whilst longer-lived states are better for high spatial resolution. With careful experimentation, very fast blinking rates to capture events on the microsecond scale in live cells could be achieved – already, it has been shown that ATTO655[44,47] can be made to fluoresce many times per second, with a much faster blinking rate than with the original dSTORM conditions. It is also conceivable that spatial and temporal experiments could be carried out on the same sample, assuming that there was little photobleaching of dye, simply by performing imaging experiments with different concentrations sequentially.

Whilst the most of the redox agents currently employed are certainly rather toxic and therefore limited to the imaging of fixed cells, it is hoped that suitable redox pairs for live-cell imaging can be found. Already, it can be assumed that ascorbic acid and Trolox are amongst the more benign reducing agents available, but finding inoffensive oxidants is more of a challenge. Tinnefeld's group reported the use of Trolox-quinone[47] as an oxidant; this is a particularly exciting development, as the quinone is the oxidised form of Trolox, raising the possibility of being able to use a single reagent for both the reducing and oxidising processes.

2.3 CHOICE OF DYE FOR SUPER-RESOLUTION

Quality of images produced from experiments –					
Compound	Reduction potential (V)	PBS only	Thiol	Oxygen scavenging	Thiol + oxygen scavenging
Cy3	-1.00[a]	Poor	Poor	Good	Good
Cy5	-0.88[a]	Poor	Good	Poor	Very good
Cy7	-0.72[a]	Poor	Good	Poor	Very good
ATTO647N	-0.64[b]	Good	Poor	Poor	Good
Fluorescein	-0.57[c]	Poor	Good	Good	Good
ATTO655	-0.42[b]	Good	Good	Good	Very good

***Table 1 : Evaluation of fluorophores adapted from Dempsey* et al.[48] *to include reduction potential of dyes. Potentials taken from (a) Stein* et al.[41] *(b) Doose* et al.[49] *(c)Rao and Hayon*[26]**

As techniques such as dSTORM can now be carried out with almost any fluorescent dye, there is a need to evaluate dyes in terms of their performance. An excellent review by Dempsey *et al.* from 2011[48] lists 26 popular probes and tests their performance for blink imaging under different conditions: in a buffer solution with no additives; in a solution with only an oxygen scavenging system; in a solution with only a reducing agent; a solution with both oxygen scavenging system and reducing agent. The probes tested include cyanines, rhodamines and oxazines, and all but one blink adequately under the standard blink conditions of depleted oxygen and a reducing agent in aqueous solution. Whilst the reduction potential is not mentioned by Dempsey in his study, interestingly it seems that there is a correlation between the reduction potential of the fluorophore used and its super-resolution imaging performance. Dyes with higher-lying reduction potentials seem to be suited to super-resolution imaging across a wider range of conditions. As has been suggested elsewhere, it is possible that a dye's reduction potential should be a criterion for its use in imaging. An exhaustive list of the reduction potentials of dyes is not available however, so it would be of interest if the potentials of popular dyes could be recorded.

3. LABELLING METHODS

Super-resolution techniques rely on consistent and reliable sample labelling methods. As the resolution of stochastic techniques is limited by the distance between dyes, is it important to choose a labelling method that ensures a high density of markers on the sample. There is no shortage of fluorescent dyes available, the first such markers having been introduced in the late 19th century to study biological samples.[50] These 'stains', as they are often known, bind directly to DNA through non-covalent interactions. Stains often lack specificity when binding to a sample however, and it is for this reason that labels covalently bound to specific oligonucleotides or even sequences *via* a linking unit have found application in the last fifty years. The latter group are commonly called simply labels or tags.

The ease with which non-covalent dyes can be used – labelling often requires a solution of the dye to be dropped onto the sample – means that they are still the preferred option in many cases, although it has been shown that bonding to DNA exhibited by some stains affects the integrity of the structure of DNA.[51] The structure of DNA is often less affected by covalent tags than by traditional stains, although it has been suggested that some covalently-bound probes have a flexible enough linker to allow them to interact with the DNA grooves.[52]

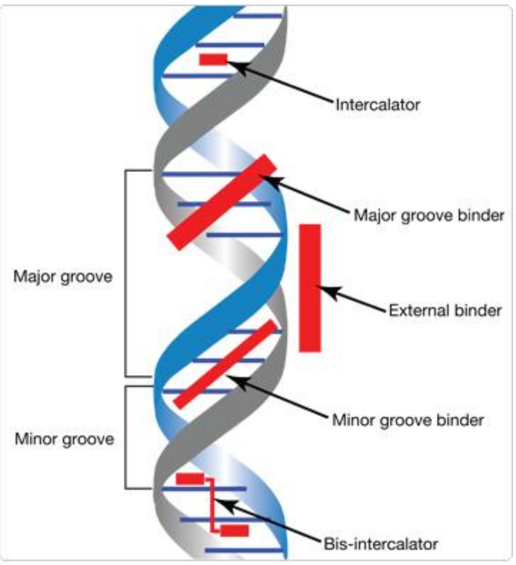


Figure 15 : DNA binding modes. Reproduced from www.invitrogen.com[53]

3.1 DNA BINDERS

The shape of the DNA double-helix as it twists gives rise to small and large gaps, or minor and major 'grooves' that can accommodate various types of molecules. Additionally, molecules can sit in the gap between base pairs in what is known as an intercalating binding mode. Some groove-binding and intercalating dyes are also known to bind externally to DNA, but this is usually a minor binding mode. Various factors affect whether a molecule will sit in a groove or between base pairs, but the most important are the size of the molecule, its flexibility and whether any of its moieties can form hydrogen bonds with the functional groups present in DNA base pairs (namely the 3, 6 and 7 positions of the purines, and positions 2 and 4 in the pyrimidines, figure 16).

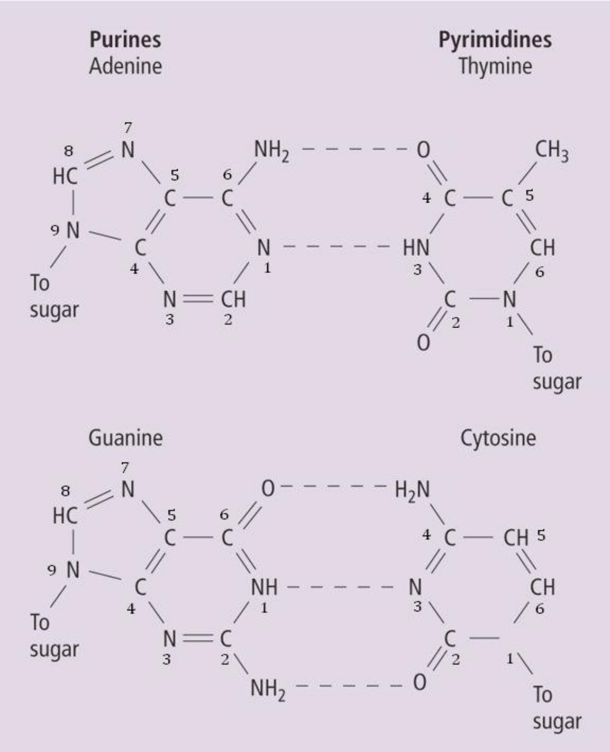


Figure 16: DNA base pair structures. Adapted from Tobias **et al.**[54]

Many DNA stains bind to different base pairs selectively: notably, the minor-groove binding Giemsa stain binds preferentially to GC-rich regions of DNA, which is the reason for 'banding', or the appearance of dark bands on Giemsa-stained chromosomes.[54] The AT-rich regions of DNA take up less of the Giemsa stain and so appear lighter under a microscope. The 'G-bands' resulting from Giemsa staining have been used with great success to aid karyotyping (the classification of chromosomes according to size) since the introduction of the protocol in the early 20th century, as each different chromosome has a distinctive banding pattern. It is possible to achieve reverse banding (R-banding) by denaturing the DNA through heating. This relies on the different melting points of the base pairs; the GC-regions denature at lower temperatures than the AT-rich regions, and so after heating of DNA Giemsa will bind to AT-rich regions. A similar effect can be achieved using dyes that bind preferentially to the AT-rich regions, such as DAPI and the Hoechst dyes.

Species that bind exclusively to the outside of the DNA helix do so because of electrostatic interactions with the charged phosphate backbone. These external binders are usually limited to species containing a metal cation, which is attracted by the negative charge on the phosphate group. One example is chlorophyll *a*, with a Mg^{2+} cation in complex with a porphyrin ring.[55]

DNA-binding proteins tend to bind to the major groove of DNA; base pairs are more exposed in the major groove than in the minor groove, and so sequence-specific proteins can more easily form hydrogen bonds with the relevant base molecules. Other major-groove binders include a range of antibiotics and anti-cancer drugs including the aminoglycosides and ditercalinium, which are designed to exploit the alternating hydrogen bond character of the different base pairs, as in proteins.[56]

Figure 17 Karyotype of human female chromosomes with G-banding; reproduced from Tobias et al.*[54]*

Most important chromophoric or fluorescent nucleic acid stains are minor-groove binders or intercalators, or in some cases display characteristics of both binding modes. Minor groove-binding molecules tend to be small and flexible in order to bend and fit into the smaller groove,[57] whereas intercalating molecules usually contain planar heterocycles which interact with the planar DNA bases, stacking on top of them. In order to encourage photon absorption, chromophores and fluorophores tend to be based on planar aromatic systems with delocalised electrons and can easily intercalate in between base pairs. Accordingly, the rhodamines and other dyes derived from the fluorescein structure (figure 18) are intercalators. Many members of another popular class of dyes, the cyanines, also intercalate, however it should also be noted that many cyanines contain a protonated nitrogen heterocycle – the associated positive charge means that there is an additional interaction with the phosphate backbone of DNA.[57]

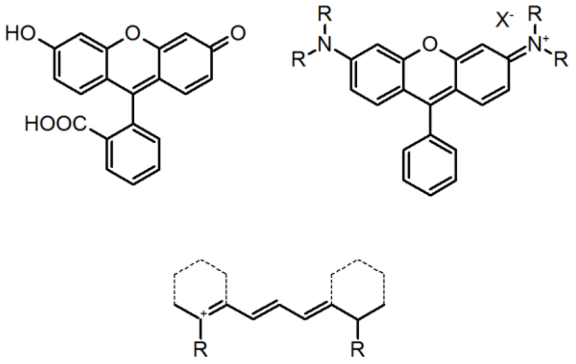


Figure 18 : Top left, the fluorescein structure; top right the rhodamine structure, bottom; the cyanine structure

Some modern unsymmetrical cyanines designed to bind to DNA, such as PicoGreen and the thiazole orange (TO) family of dyes, contain amine groups (not part of the delocalised electron system) which hydrogen-bond to the nucleobases in DNA. These amine moieties, combined with the planar fluorophore group in the dye, mean that the aromatic part of the cyanine intercalates whilst the amine 'arms' lie in the minor groove in a 'bis-intercalating mode' (figure 19).[58] Bis-interlacation is also observed for the TOTO and YOYO dimers, with the planar aromatic groups intercalating at different sites and linked by the long quaternary-amine-containing linking chain lying in the minor groove.[59,60] It is therefore not uncommon in studies to see that some molecules show similar characteristics to both intercalating and minor-groove binding dyes in experiments. Generally the binding mode of a dye can be studied using 1D NMR to detect shifts in the DNA spectrum compared to existing assignments for specific sequences, circular dichroism to detect the difference in polarisation of light from free fluorophores and those bound to chiral DNA, and viscometry experiments, which can ascertain the extent of lengthening of DNA due to intercalation.[57]

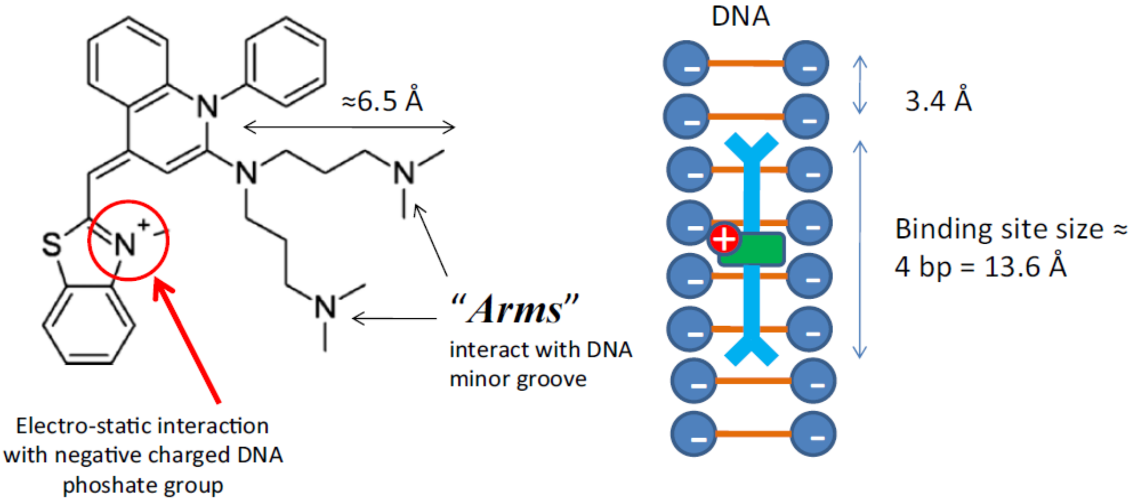


Figure 19 Structure of PicoGreen and schematic diagram of its DNA binding mode; reproduced from Dragan et al.[58]

It would be beneficial to carry out computational studies of dye-DNA interactions to determine, amongst other things, the extent of charge transfer and the extent to which the DNA structure is disrupted upon binding of a dye. Charge transfer is particularly important with respect to the absorption and emission wavelengths of fluorophores, and also the reduction potential of the molecules plays an important part in the formation of fluorophore dark states so it would be useful to confirm experimental observations for popular fluorescent dyes. DFT studies reported so far tend to focus on the interactions of drugs with DNA,[61] but it is possible that if computational studies are able to accurately model experimental observations, they could be used to screen new fluorescent probes for use as DNA stains.

Molecular dynamics studies[62] have shown how rhodamine 6G interacts with base pairs, however DFT studies are needed to determine the electronic properties of systems under observation.

3.2 LABELS AND TAGS

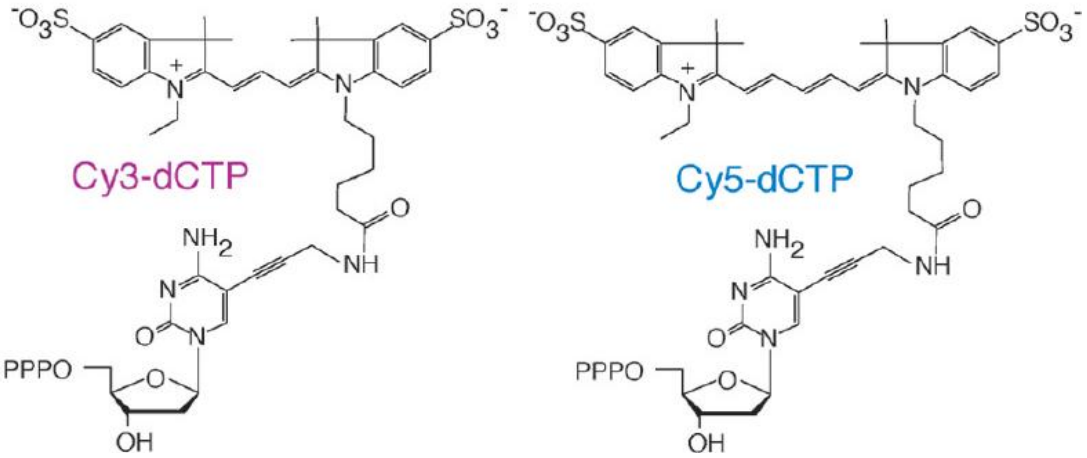


***Figure 20 Cy3 and Cy5DNA is formed by linking fluorophore-tagged nucleotide analogues using a polymerase; reproduced from Ramsay* et al.[66]**

The replacement of nucleobases in DNA with custom-made analogues is not a new concept, with such techniques having been used to monitor DNA synthesis or to model DNA structures for last couple of decades.[63,64] Classically, this involves the incorporation of nucleobases linked to a bulky fluorophore *via* an alkyne group using specific enzymes; examples include nick translation[65] using an *E. coli* polymerase and the CyDNA protocol[66] using a *P. furiousus* polymerase (figure 20). Nick translation has been used since the 1970s, whereas the enzyme used for CyDNA was isolated only in 2010. Such enzymatic techniques are useful, however the bulkiness of the fluorophore affects how well DNA can be synthesised. As such, with nick translation techniques, the length of DNA labelled is limited to around 100bp, whilst the CyDNA protocol allows for fragments of up to 1000bp to be labelled. Both techniques have therefore found applications in the synthesis of probes for Fluorescence In-Situ Hybridisation,[67] which requires the labelling of different genetic sequences within a chromosome.

With the advent of so-called 'click' chemistry, however, the labelling of DNA has entered a new realm. Click chemistry[68] describes reactions that have quantitative yields, generating by-products that are inoffensive and which are carried out using readily-available reagents with no solvent, or at least a solvent which is easily removed. Modified nucleobases can be incorporated into DNA during active transcription before fixing the sample; these nucleobase analogues have appropriate functional moieties to which fluorophores can be attached afterwards using a simple ‘click’ reaction. There are several advantages to this technique, not least the fact that the base analogues lack the bulkiness of those used in the synthesis of CyDNA, and can therefore be used in live cells. Furthermore, the number of bases that can be labelled is not limited as for the enzymatic techniques, so entire cells can be labelled with nucleobase analogues.[69]

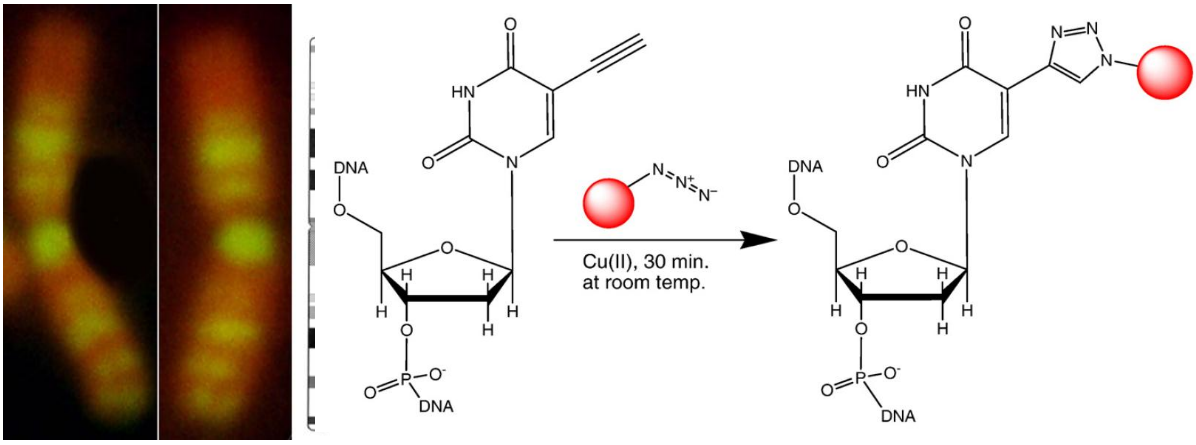


***Figure 21 Left, R-type banding using Alexa-488-taggeed EdU (green) with a YOYO-3 counterstain (red), reproduced from Hoshi and Ushiki.[67] Right, the click chemistry protocol using EdU, reproduced from Zeng* et al.[66]**

A click reaction that has been commercialised for use in DNA labelling is the azide-alkyne Huisgen cycloaddition,[70] whereby a coupling is formed by the copper (I)-catalysed reaction of an azide group with a terminal alkyne (figure 21), a reaction that occurs in aqueous solution at room temperature. This reaction is particularly useful within the context of DNA labelling, as a modified nucleobase substituted with either an azide group or a terminal alkyne can readily be incorporated into the DNA backbone.[71] Once the cell culture has been achieved, a fluorophore with the appropriate corresponding functional group can be attached to the modified DNA *via* the Huisgen cycloaddition. Another important point is that the labelling is inherently base-specific; appropriate modified analogues exist for each of the nucleobases, so it would theoretically be possible to label each of the base pair types in DNA with a different fluorophore, so as to differentiate between AT- and GC-rich areas. At the time of writing, such an experiment has not been attempted, however one paper exists where chromosomes containing modified 5-ethynyl-2’-deoxyuridine (EdU) nucleobases were click-labelled with Alexa-488 fluorophores and counterstained with YOYO-3 were shown to display banding patterns.[72]

This result demonstrates an important proof-of-concept for the labelling of chromosomes using modified nucleobases and click chemistry: the fact that practically any fluorophore can be modified to perform a click reaction bodes well for the super-resolution imaging of chromosomes using this labelling technique. More experiments are needed to ascertain whether the replacement of multiple bases within the DNA structure with species modified for click chemistry is possible. Furthermore, it would be advantageous to have more click reactions at our disposal so that we are not limited to two fluorophore types – an RGB system, for example, could produce some rather interesting and maybe even beautiful results.

4. CONCLUSIONS AND OUTLOOK

Optical super-resolution techniques are well established and have successfully been used to generate striking images of biological samples. Indeed, some predict that super-resolution images will become a staple part of many biological papers as a result of the commercial setups being offered by manufacturers. Super-resolution is for the moment only suited to some samples, mainly because of the harsh sample preparation required. It is certain that the next developments in stochastic techniques will involve steps towards real-time live-cell and 3D imaging. Whilst the latter will be dependent on innovations in optical systems, research into real-time imaging will focus on dye design and the improvement of ROXS systems, and also on the software used to reconstruct images from the vast amounts of data generated during experiments.

The main barrier to real-time imaging is currently the speed of acquisition of images, which is governed by the blinking rate of fluorescent markers. Whilst certain ROXS systems allow fluorophores to blink up to ten times per second, the fact that thousands of frames are required for an adequate image to be reconstructed means that image acquisition is still takes place on the order of minutes. Faster-blinking dyes would reduce this time, and therefore a suitable goal would be to increase the blinking rate of dyes to 1000Hz, and the image acquisition time to the order of seconds. This can be achieved through the design of new dyes to increase the rate of cycling from the dark state back to the fluorescent states, for example through the inclusion of heavy atoms, and by finding optimal ROXS conditions for new dyes. Furthermore, new dyes containing heavy atoms might be compatible with electron microscopy or X-ray imaging techniques, and would open up avenues into the field of correlative microscopy.

The problem of real-time imaging is not as simple as just increasing temporal resolution, however. Even with very fast acquisition times, the problem of drift would still exist, as live samples are notoriously difficult to get to keep still, and new problems such as surpassing the frame rates of even the most modern cameras would arise. These are hurdles to overcome, but given the revolutionary steps taken in recent years, there is no reason to suspect that real-time imaging will not happen in some form in the near future.

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