A Little Light Relief

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Abstract
Photomedicine as a 'modern' subject has been around since the late 1880's, and currently encompasses the effects of light upon the skin; diagnostic uses of light, therapies using non-laser light, and the use of lasers. A brief review of these is given. Effects of light on the skin include production of *Vitamin D*, tanning, ageing of the skin, and the skin cancers *basal* cell and *squamous* cell carcinomas, and *malignant melanoma*. Diagnostic uses of light include luminescence [photo and chemi] in immunoassay, fluorescence in cell sorting, and the various forms of fluorescence microscopy, including confocal, fluorescence lifetime imaging [FLIM], and single molecule. Therapies include the *PUVA* treatment of *psoriasis* and *vitiligo*, blue light curing of neonatal jaundice, photoinactivation of microbes. Laser treatments include ablative corrective eye surgery, general 'bloodless' surgery, and of most importance photochemically, the various treatments using sensitisers and laser light known as *Photodynamic Therapy, PDT*. Future developments in PDT will be through targeted PDT, in which the tissue to be destroyed receives the photosensitiser in a highly selective fashion. Strategies to achieve this highlighted here are the use of monoclonal antibody fragments selected for tumour cell targets; and two-photon spatial selection.

Introduction

Photochemistry has had an impact on society in a wide variety of ways. The fact that we exist on the planet at all is a result of the oxygen in the atmosphere provided by green plants through photosynthesis, and the resulting ozone layer in the stratosphere which provides the ultra-violet radiation shield. The food we eat ultimately comes from photosynthesis . Modern electronics relies upon photochemical polymerisations to create miniature circuitry on electronic chips. The fact that we can see involves photochemistry, the dyes and pigments we use, all are examples of photochemistry in action, many known since early civilisations. More modern examples include the many uses of lasers in our everyday lives, in supermarket tills, in theodolites, in remote sensing, in welding and cutting in industry and in dentistry, in optical treatments, and in medicine generally. In this article, we concentrate upon the uses of light and lasers in medicine.

We will outline a brief history of photo-medicine, and then describe very briefly current uses of light in medicine.[1], [2]

Historical Perspective

The father of modern photo-medicine was Neils Finsen,[Figure 1] the Danish physician who in the late 19th century used ultra-violet light to cure the facial sores [*lupus vulgaris]* exhibited by sufferers from tuberculosis. [Figure 2]. For this at first sight relatively trivial advance, he was awarded one of the first Nobel Prizes for medicine in 1903, and this brought to the attention of the world the potential medical uses of light.
In the 20th century, and particularly since the invention of the laser in 1960, light has played an increasing role in medical matters.
The present applications can be summarised briefly as follows:
[1] The effects of light upon the skin
[2] The uses of light in diagnosis, including cell-sorting and fluorescence microscopy
[3] Therapeutic treatments using non-laser light
[4] Uses of lasers.

Figure 1 Neils Finsen, the father of Photomedicine

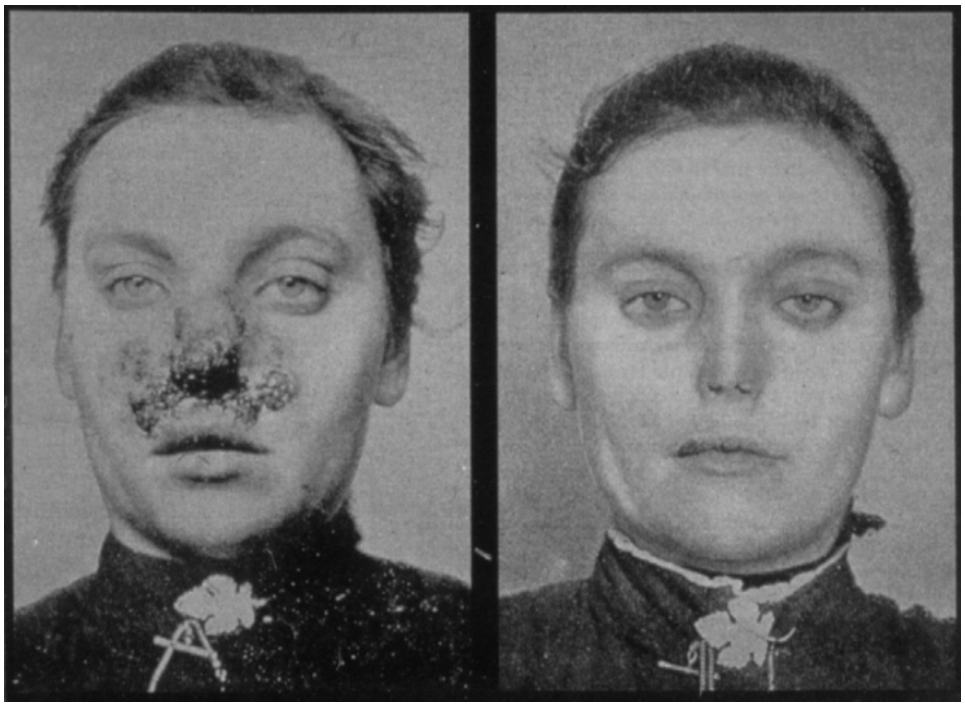


Figure 2 One of Finsen's patients, exhibiting *lupus*[left], and after uv treatment [right]

[1] **Skin**
The skin is the heaviest and most accessible organ of the body, and thus light can have dramatic effects, some harmful, some beneficial.

The obvious beneficial effect is the synthesis by sun light of *Vitamin D*, which boosts that taken in in the diet. Too little *Vitamin D* results in bone deformation, *[rickets],* due to the role of *Vitamin D* in the calcium uptake process. Rickets was very common in Victorian Britain, at the same time, was virtually unknown in India at the same time, reflecting the very different extent

of solar inputs in the two locations. *Vitamin D* deficiency has become quite common again in the UK, but is easily detected, and treated using dietary augmentation.

The negative aspects of light upon the skin are premature ageing, and skin cancers.
The former effect is dramatically illustrated by the two photographs of Brigitte Bardot, as an 18 year-old film starlet, and very recently.[Figures 3] The loss of elasticity of the skin is obvious, and this is attributable solely to cumulative exposure to ultra-violet light.

Figure 3 [a] Brigitte Bardot, aged 18, and [b] recently

Far more sinister than ageing is the dramatic increase in skin cancer which has become endemic in the Western world, due to the modern habit of exposing the body to large amounts of ultraviolet, either from natural sunbathing, or the increased use of ultra-violet tanning salons. Skin cells are produced in the basal layer, [*basal cells*] and travel towards the surface [*squamous cells*], becoming less globular, until they reach the surface [28 days after cell birth, in a normal human], where they are flat dead flakes, which are lost to the environment [much household dust is the dead skin cells of inhabitants]. Some cells have melanin pigment, [*melanocytes].* Tanning represents the reaction of the skin to the ultraviolet threat, and thus should be treated with caution, ie the use of sun-blocking and attenuating agents. All cell types can become malignant under the influence of ultra-violet light. *Basal cell* and *squamous cell carcinomas* do not in general metastasise, so can be treated locally, usually surgically.

Malignant melanoma undergoes rapid metastasis, and should be treated immediately to avoid development of secondary, and usually fatal, metastatic cancers in other parts of the body. Treatment using photodynamic therapy will be discussed later.

[2] Light in diagnosis

Luminescence, both chemiluminescence and fluorescence is in widespread use for immunoassay, ie, the identification of antigens in the body which may be precursors to disease. The immunometric assay uses a combination of two antibodies, one immobilised which is specific to the antigen being tested for, the second, the labelled antibody, which binds to a different site on the antigen. When a fluid sample, eg blood, urine][is tested, if the antigen is present, it binds to the immobilised antibody, and this permits the labelled antibody to bind, ie the system is positive-reading. If the antigen is absent, the second antibody cannot bind, and so there is no luminescent signal. The signal can be activated by addition of peroxide in the case of chemilumiunescent labels; by light for fluorescent labels. Examples of the use of immunoassay might be the testing for pregnancy at early stages by seeking the hormone *human chorionic gonadotrophin,* or testing for the *HIV* virus, but he technique is ubiquitous.

Fluorescence microscopy has become a universally used tool in biology and medicine, particularly confocal microscopy, and latterly, fluorescence lifetime imaging microscopy, FLIM,[3] and most importantly, single molecule microscopy and techniques such as stimulated emission depletion, STED [4];local-depletion microscopy RESOLFT, and photo-activation localisation microscopy, PALM [5,6] Examples of our own work in FLIM are discussed here, as an indicator of the increasing use of these techniques in biological studies with medical applications.

FLIM systems are usually based on femtosecond lasers, and a pioneering wide-field instrument developed by colleagues in Imperial College is shown in Figure 4 [7] The instrument has been used in many biological studies, including studies on the immune system, in particular, investigation of the interaction between the *major histocompatibility complex*, *MHC*, and *natural killer cells, NKC*.[8,9] Figure 5. The primary interrogation of proteins in cells is carried out by *T-cells* on cellular proteins exhibited by the *MHC*; alien proteins result in the destruction of the cell by the immune system. Some viruses are able to remove the *MHC*, thus the *T-cells* do not recognise these cells as infected; the *NKC* interacts with the *MHC* directly, and its absence then triggers the destruction of the cell. Extensive studies have been carried out on the interaction between the *NKC* and suspect cells using *MHC* labelled with *Green Fluorescent Protein* [*GFP*].

Figure 4 FLIM instrument [10]

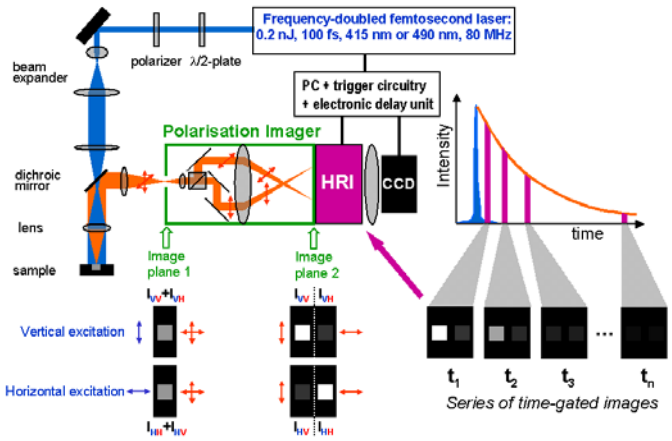


Fig. 1: (left) The experimental set up of the picosecond time-domain TR-FAIM and FLIM instrument. The Polarization-Resolved Imager (PRI) containing a polarizing beamsplitter is situated directly in front of the gated optical image intensifier (HRI), the output screen of which is imaged with a CCD camera. The PRI splits a single image (here, a single square well of a multiwell plate) in image plane 1 by its polarisation components into two images, spatially identical but showing different polarisation components (image plane 2). (right) A series of time-gated fluorescence intensity images containing both polarisation components are acquired by sampling the fluorescence decay at a range of delays after the excitation pulse. The time-resolved fluorescence images for parallel and perpendicular polarization are recorded simultaneously.

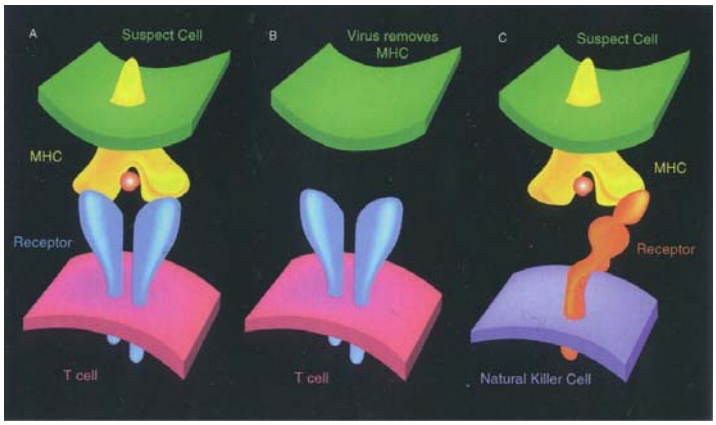


Figure 5 Cartoon of interaction between *T-cells* and *Natural Killer Cells* with suspect cells.

The details of theses studies are beyond the scope of this article, we were able to observe a considerable difference in the fluorescence lifetimes of a fluorescently labelled *MHC* constitutively expressed at the cell surface, and clustered at the immune synapse. The primary cause of the change in lifetime may be the local refractive index,[11] changing possible because of changes in lipid content or protein concentration. The application of new physical techniques to important problems in cell biology is often the path to unexpected discoveries, as outlined briefly above, where micron scale supramolecular organisation of *T-cell* and *integrins* at immunological synapses were discovered by the use of 3-D fluorescence microscopy. Imaging such fluorescence parameters as lifetime, spectrum, anisotropy, and

use of *Forster Resonance Energy Transfer, FRET*, will certainly reveal new aspects of inter and intra-cellular communication.

A further variable in the study of cells is the local viscosity, which is believed to exert considerable physiological effect.[12] The system described in Figure 4 is capable of measuring directly the fluorescence anisotropy decay in living cells, and has been used for this purpose. An alternative to this direct measurement is the use of probe molecules where a considerable dependence upon the fluorescence yield and decay time upon local viscosity is exhibited. BODIPY molecules are good examples of this type of probe, and results showing variations in viscosity in sample cells incubated with the probe are shown in Figure 6.[13]

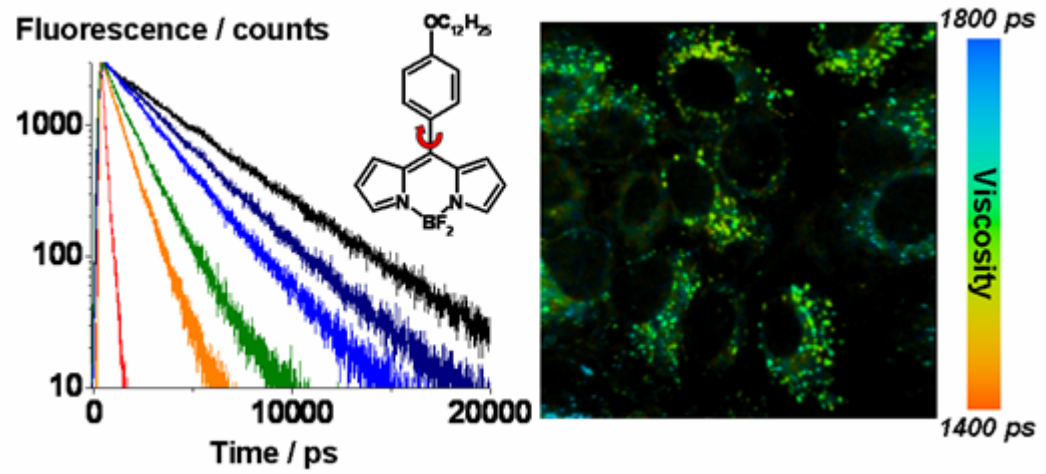


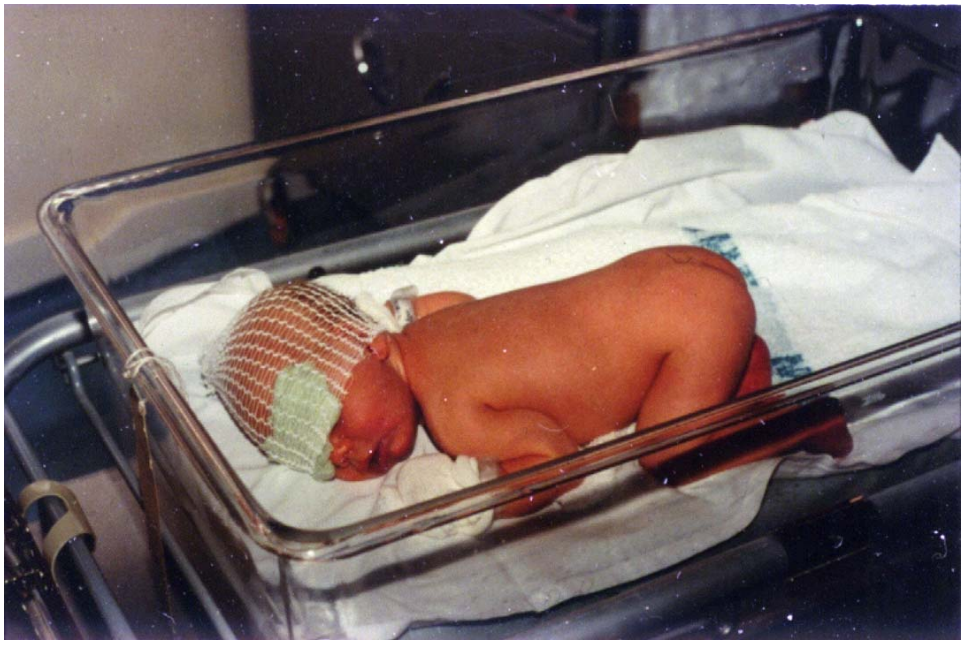
Figure 6 False colour images of cells incubated with the probe shown, and resulting fluorescence decay times, illustrating intracellular variations in viscosity.

[3] Therapy

Uses of non-laser light in therapy
[i] Neonatal jaundice [1,2]

Since the late 1950's, neonatal jaundice has been treated using blue light. The condition arises in premature infants, and those born with minor liver malfunction, which is very common. Breakdown of red blood cells in the body produces copious amounts of the yellow porphyrinic pigment *bilirubin*, which is water insoluble, but dissolves readily in fat. Normal adults have an enzyme in liver and bile which converts this fat-soluble form of *bilirubin* to a water-soluble form, which permits excretion of the *bilirubin* in urine and stools. Before birth, the foetus does not have the enzyme, since it cannot excrete; immediately after birth, the enzyme is needed, and usually develops with a day or so. For those premature infants the development of the enzyme may take much longer, and the fat soluble bilirubin is stored in the skin, hence the jaundice. A chance observation in the UK in the 1950's of a jaundiced infant in sunlight who became bleached led to the widespread use of visible [blue] light to treat the condition. Figure 7 shows my nephew receiving treatment. The mechanism was widely disputed until recently, but the consensus view now is that the *bilirubin* molecule under the influence of light undergoes a photochemical cis-trans isomerisation about one of the double bonds in the structure [Figure 5], revealing a water-solubilising carboxylic acid group which is buried internally in the water insoluble structure. This mechanism is supported by the fact that the photochemical reaction is very fast, thus a concerted process.

Figure 7 Infant receiving light treatment for *hyperbilirubinaemia*



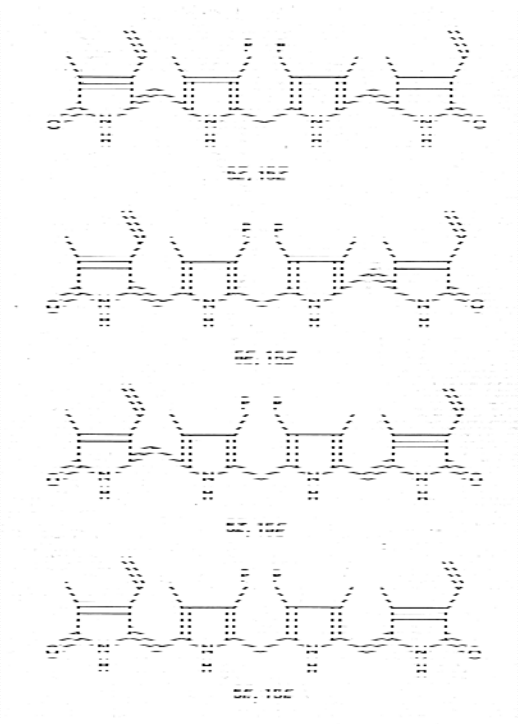


Figure 8 Structures of bilirubin

[ii] *Psoriasis*

In the various forms of *psoriasis*, the basal layer of the skin overproduces skin cells, such that when they travel to the surface they are still viable. The treatment is to use a sensitising agent a fumocoumarin, *psoralen,* which absorbs near ultraviolet light and destroys the adjacent skin tissue The term used for the therapy is the *PUVA* treatment. The treatment does not address the cause of the disease, merely treats the symptom, but affords much relief to patients

[iii] *Vitiligo*

The same treatment can be used to treat *vitiligo*, the loss of melanin in pigmented skin cells, resulting in white patches. These are not much of a problem in Caucasian skins, but are very disfiguring in pigmented skins.

[4] Uses of lasers

The effects of laser light upon human tissue depends upon the fluence, and the presence or absence of a sensitiser. At low levels, <4 watts/sq cm, the effect is to stimulate development of cells, a process used in some countries to assist wound healing. At higher energies, the effects are the opposite, to slow down cell growth or even destroy tissue. Thus at light levels of 40 watts per sq cm, the light alone has little effect, but in the presence of sensitisers, can lead to tissue destruction, one form of which is termed photodynamic therapy, [PDT] discussed below in detail. At 400 watts/sq cm, photo-thermal effects dominate, with sealing of blood vessels a desirable goal [photocoagulation], and for very powerful lasers, >4000 Watts/sq.cm, tissue ablation occurs. The precise details of these processes are dependent upon the wavelength of the laser, and whether pulsed or continuous. Present day medical uses include [i]] Laser surgery, the use of a powerful, usually ir YAG laser the cut tissue. The laser cauterises as it cuts, thus leading to bloodless surgery. The treatment is often only a palliative in later stages of disease, such as upper respiratory tract tumours, as illustrated in Figure 6, where a tumour at the branch of the junction of the trachea and bronchi is removed by laser, giving a better quality of life for the patient, whose condition is terminal in any case due to metastatsic tumours elsewhere.

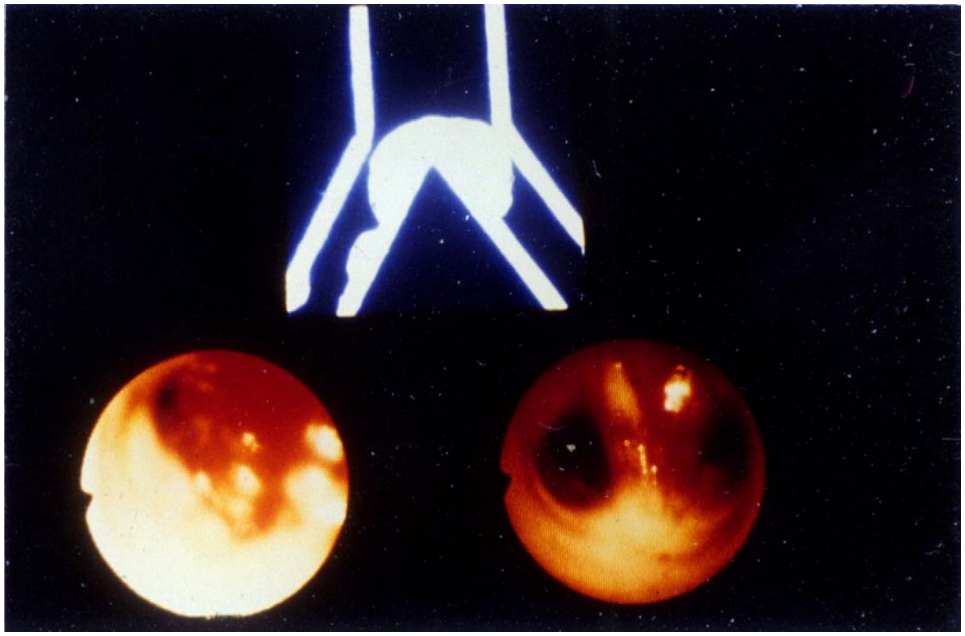


Figure 9 Removal of upper respiratory tract tumour tissue by ir laser [Courtesy of University College Hospital]

[ii] Eye treatments, particularly laser eyesight corrective procedures. Here an ablating ultraviolet laser, usually an excimer, short wavelength source, is used to reshape the cornea of patients with defective vision, thus permitting the lens to focus visible light accurately upon the retina. The laser is chosen since it has an extremely short penetration distance, thus is not a danger to the retina. Such treatments are generally extremely effective, and for most patients, painless.

[iii] Photodynamic therapy

It is the author's belief that it is in the development of PDT, and its increasing use in medicine, that the main future of photomedicine lies, and so the remainder of this article is concentrated upon this topic. Photodynamic Therapy (PDT) is a minimally invasive procedure used in treating a range of cancerous diseases,[14] infections [15] and recently, in ophthalmology to treat the wet form of *age-related macular degeneration* (AMD). [16] The photodynamic action relies on the simultaneous interaction between a

non-toxic photosensitiser molecule, visible light and molecular oxygen, offering dual selectivity through preferential uptake of the photosensitiser by diseased cells and the selective application of light. Following activation with visible light of the appropriate wavelength, the photosensitiser generates reactive oxygen species (ROS), primarily the reactive singlet state of molecular oxygen, called singlet oxygen, $O_2(a^1\Delta_g)$, through the energy transfer to the ground state triplet oxygen, $O_2(X^3\Sigma_g^-)$. Other photochemical products of energy and/or electron transfer include radicals, *e.g.* superoxide anion $O_2^{-\bullet}$ and hydroxyl radical $OH^\bullet$. Production of these short lived species within biological tissues leads to localised cell death *via* irreversible damage to cellular components such as proteins, lipids and DNA.

The limited diffusion distance of ROS in biological systems means that diffusion only occurs at the intracellular level and furthermore, that the primary site of ROS generation determines the first point of damage to the cell. Consequently, the subcellular localisation as well as the selective accumulation of photosensitisers in diseased cells are important factors in determining PDT efficacy. Although the production of singlet oxygen is widely accepted as the dominant mechanism in PDT, it should be stressed that electron processes may play a role [17]

A key component of PDT is the photosensitiser which has to possess a number of key properties including absorption in the red (600-800 nm) allowing photoactivation within deeper tissues, selective uptake by malignant cells, the ability to efficiently generate singlet oxygen and minimal dark toxicity. Since the clinical approval of Photofrin®, a first-generation photosensitiser with a number of limitations such as prolonged skin photosensitivity and poor absorption in the red, efforts have concentrated on so-called second generation photosensitisers with substantially improved properties over Photofrin®. A majority of these second generation photosensitisers are based on modified tetrapyrrolic macrocyles (porphyrinoids) with excellent absorption profiles at longer wavelengths and include both naturally derived and synthetic molecules, such as Photosens [a trisulphonated phthalocyanine], Foscan, [a bacteriochlorophyll], Visudyne, and other chlorins, bacteriochlorins, benzoporphyrin derivatives and naphthalocyanines. The use of Photosens to treat a basal cell carcinoma in a patient is shown in Figure 12, where the benefits as an alternative to potentially disfiguring surgery are obvious.

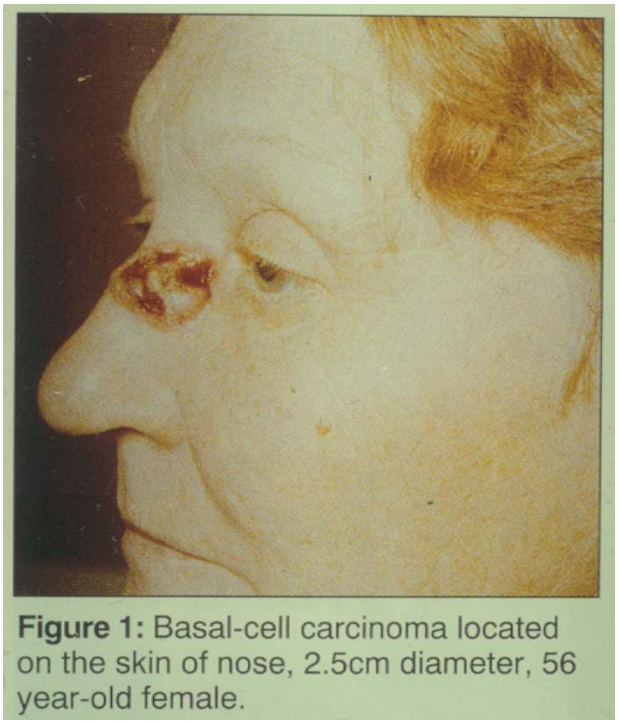


Figure 1: Basal-cell carcinoma located on the skin of nose, 2.5cm diameter, 56 year-old female.

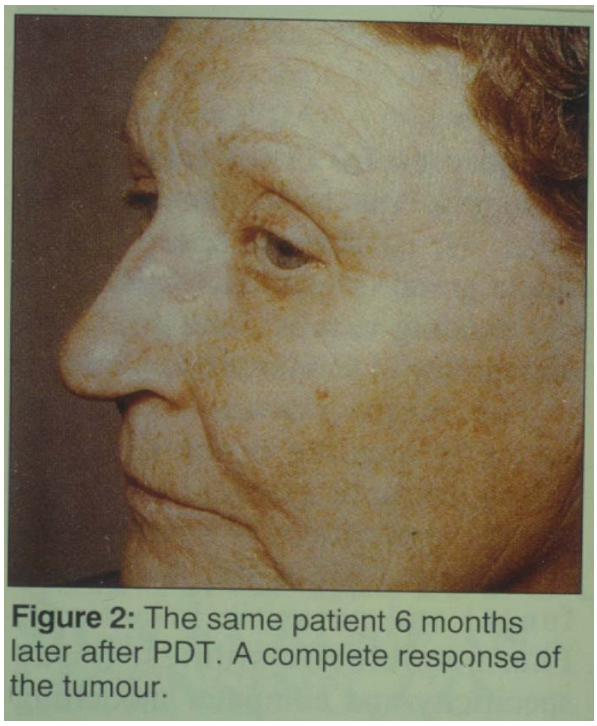


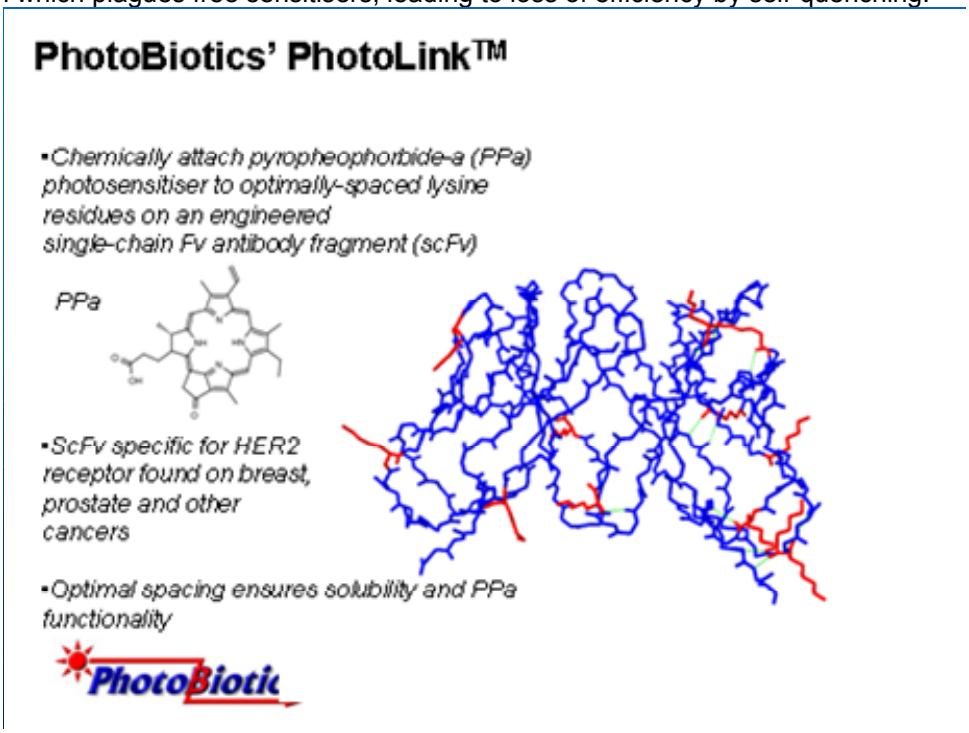
Figure 2: The same patient 6 months later after PDT. A complete response of the tumour.

Figure 12 [a] patient with basal cell carcinoma [b] same patient after PDT with aluminium phthalocyanines sensitised PDT.

A recent count showed that there are in excess of 1450 molecules identified in the literature as being potentially of use in PDT. Very few of these will be used for this purpose, due to the very high cost of introduction into the clinic associated with Phase I , II and III clinical trials. Only those sensitisers with exceptional properties will negotiate these financial hurdles. Since the currently approved sensitisers have quantum yields of ROS formation generally of 0.5 or above, the maximum benefit that can be reached with new sensitisers based upon photochemical performance alone is a factor of two, unless a photochemical mechanism involving chain oxidation can be achieved. It is thus the belief of this author that choice of new photosensitisers will be based upon their biological properties rather than photochemical, and

that key to this is the targeting of the sensitisers to the tissue to be destroyed. Currently used free sensitisers achieve targeting only in the ration of 2-5:1 tumour to normal tissue; improving this by at least an order or magnitude could reduce dramatically the dosage required for the PDT effect, and reduce considerably side effects such as skin sensitivity.

The principal means to achieve targeting include
[i] Whole antibodies
[ii] Monoclonal antibody fragments
[iii] Peptides, sugars, folic acid.
[iv] Multifunctional nanoparticles.
[v] Spatial targeting using two-photon excitation.
Our own work has concentrated upon single chain monoclonal antibody fragments on which typically eight to ten sensitisers per monoclonal can be achieved, without causing the aggregation which plagues free sensitisers, leading to loss of efficiency by self-quenching.



[Figure 13]
Figure 13 PhotoBiotics monoclonal antibody fragment with covalently linked pheophorbide a sensitisers.

The sensitisers are attached to the monoclonal antibody fragments via a peptide linkage to the lysine amino acids in the monoclonal. For some antibodies, lysines in the binding pocket have thus to be genetically altered so as to prevent attachment of a sensitisers in this location, which would eliminate specific binding. The details of the chemistry associated with the attachment of the sensitiser to the monoclonals, the choice of targets, and comparison with existing sensiitisers are all outside the scope of this article, but a typical result of this type of targeted PDT is shown in Figure 14, in which a thrice-repeated light treatment on mice bearing a human carcinoma is shown to be completely successful in the case of the monoclonal antibody-sensitiser conjugate in eradicating the tumour, whereas the free sensitiser merely arrests growth for a time before re-growth occurs. This result has been repeated with a variety of targets for different tumours, and a range of different sensitisers, and in the view of the author, provides the prospect of a real advance in the appeal and efficacy of PDT.

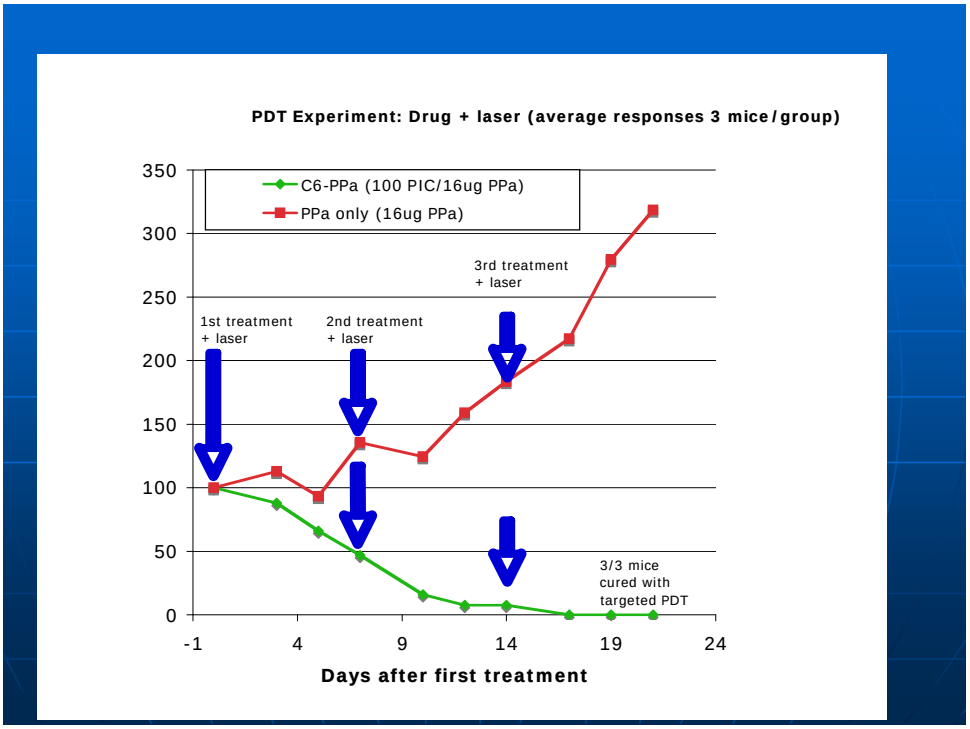


Figure 14 PDT treatment of nude mouse with human carcinoma using [upper curve], free pheophorbide a [PPa] sensitiser;[lower curve], the PPa-monoclonal antibody conjugate]

The second approach to targeting used in the authors laboratories is that of two-photon excitation, such that it is only at the focal point of a pulsed laser that there is sufficient intensity for the two-photon process to occur. The effects are thus spatially confined and thus are controlled by the focusing of the laser Two-photon excitation has the advantage of using red or infra-red light, which penetrates tissue much more readily that visible light needed for one-photon excitation. The two-photon process may thus have some potential as a means of achieving spatial selectivity in PDT,[18,19] though it must be admitted that there are practical difficulties associated with focusing lasers within highly scattering media such as human tissue. Nevertheless, the principle has been demonstrated by the two-photon PDT sealing of blood vessels in mice. [20].

CONCLUSIONS

Light plays a perhaps surprising role in modern medicine, and through laser surgery, eye-correction, and particularly targeted PDT, the uses will grow.

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