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**Wavelengths and temporal effects on the response of mammalian cells to UV radiation: limitations of action spectra illustrated by genotoxicity.**

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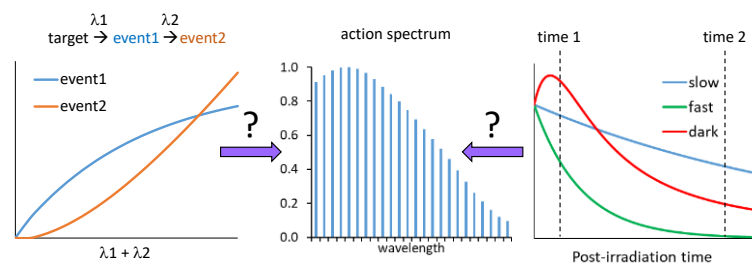
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## Highlights

1. Cellular responses to UV are strongly wavelength-dependent
2. Action spectra describe the extent of a response at different wavelengths
3. Non-additive effects may prevent use of action spectra
4. Temporal effects has to be considered when using action spectra
5. New mathematical tools are needed to study the effects of complex UV sources

## Graphical Abstract



## **Abstract**

All photobiological events depend on the wavelength of the incident radiation. In real-life situations and in the vast majority of laboratory experiments, exposure always involves sources with various emission spectra spreading over a wide wavelength range. Action spectra are often used to describe the efficiency of a process at different wavelengths and to predict the effects of a given light source by summation of the individual effects at each wavelength. However, a full understanding of the biological effects of complex sources requires more than considering these concomitant events at each specific wavelength. Indeed, photons of different energies may not have additive but synergistic or inhibitory effects on photochemical processes and cellular responses. The evolution of a photobiological response with post-irradiation time must also be considered. These two aspects may represent some limitations to the use of action spectra. The present review, focused on mammalian cells, illustrates the concept of action spectrum and discusses its drawbacks using theoretical considerations and examples taken from the literature. Emphasis is placed on genotoxicity for which wavelength effects have been extensively studied. Other effects of UV exposure are also mentioned.

**Keywords:** wavelength-dependence, skin cancer, action spectra, non-additive effects, models,

## **1. Introduction**

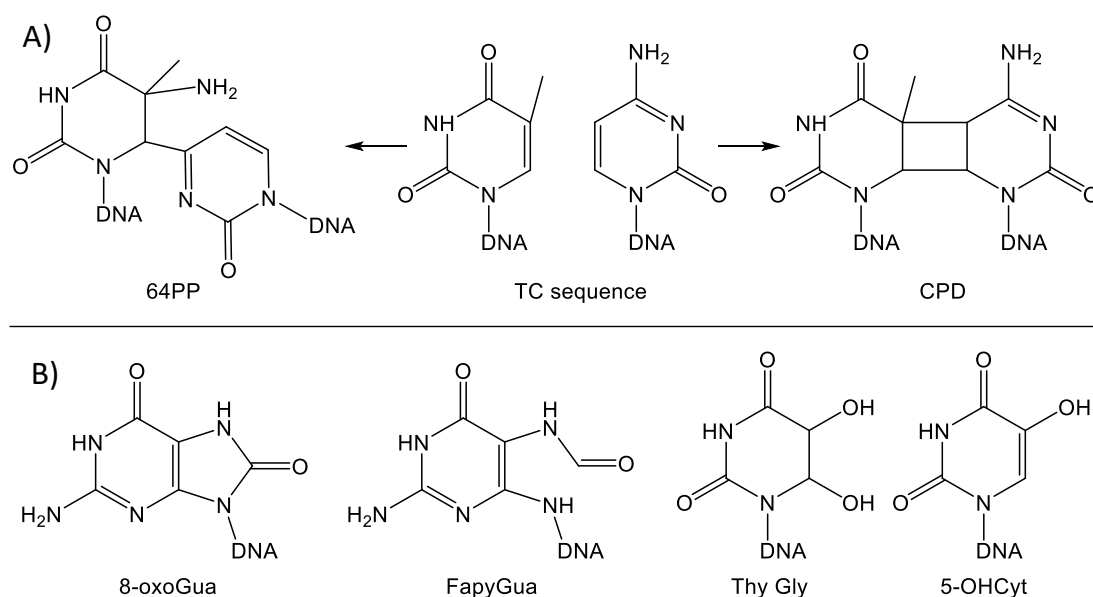
Sunlight exhibits some positive aspects for humans such as the synthesis of vitamin D. However, overexposure to solar UV is at the origin of deleterious processes such as photoaging, impairment of the immune system and skin cancer [1, 2]. One major aspect to consider in order to understand these biological consequences is the fact that solar UV is constituted of infinity of radiations with wavelengths spreading from 290 to 400 nm, and that biological effects depend on the energy of the incident photons. This spectral distribution varies according to numerous factors such as latitude, altitude, time of the day, etc. [3, 4]. The intensity the solar UVB (280-320 nm) radiation reaching the Earth's surface also depends on the content of ozone in the stratosphere [5]. In addition to overexposure to sunlight, increasing use of artificial UV sources of various spectra such as those used in tanning equipment has added new concerns to public health [6, 7]. The issue of the diversity of UV emission spectra is also important for laboratory experiments since lamps produced by manufacturers have very different features. A major difficulty for photobiologists and photodermatologists is to link the wide variety of biological effects with the numerous UV sources exhibiting different spectra. The most commonly applied strategy is to rely on action spectra, which sum the efficiency of photobiological responses at individual wavelengths. Experimental works and physical considerations have provided guidelines on the proper way to determine these spectra [8-11]. Examples of limitations have been mostly reported for microorganisms and plants. Increasing understanding of the cellular response of mammalian cells and humans to irradiation also shows that combination effects and temporal aspects may hamper the predictive accuracy of action spectra. The purpose of this review is thus to provide an update on these different aspects through theoretical considerations and examples taken from the field of DNA damage for which spectral effects have been widely studied. Reference to other biological effects such as immunosuppression will be also made.

## **2. Formation and repair of DNA photodamage, typical wavelength-dependent processes**

Large amounts of information are available for the formation of both direct photoproducts and oxidatively generated lesions in DNA. In addition, works have reported the effects of UVB (280-320 nm) and UVA (320-400 nm) on the DNA repair efficiency.

## 2.1. Wavelength dependence of the formation of UV-induced DNA damage

The UV portion of electromagnetic radiation is an efficient mutagenic agent [12]. This is mostly explained by the accumulation of DNA damage [13, 14]. Errors occurring upon replication of damaged DNA may lead to mutational events [15] within proto-oncogenes and tumour suppressor genes and thus initiate skin cancer. The yield and nature of UV-induced DNA damage is strongly wavelength dependent. UVB very efficiently induces cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (64PP) [16-23]. The yield varies from one photoproduct to the other at the four possible bipyrimidine sequences (TT, TC, CT and CC). TT and TC CPD are the most frequent, and 64PP are roughly 3-times less frequent than CPD [24]. UVA induces CPD [18, 25-32], with a much lower efficiency than UVB, but not 64PP. Another genotoxic effect of UVA is the induction of oxidatively generated DNA lesions (Fig. 1). This occurs through photosensitization processes mediated by endogenous chromophores leading to the formation of reactive oxygen species (ROS) such as radical species and singlet oxygen. A large body of information is available on the formation of 8-oxo-7,8-dihydroguanine (8-oxoGua) and single strand breaks in the DNA of cells exposed to UVA [25, 33-36]. Pyrimidine oxidation products have been shown to be minor products compared to guanine damage [25, 36].



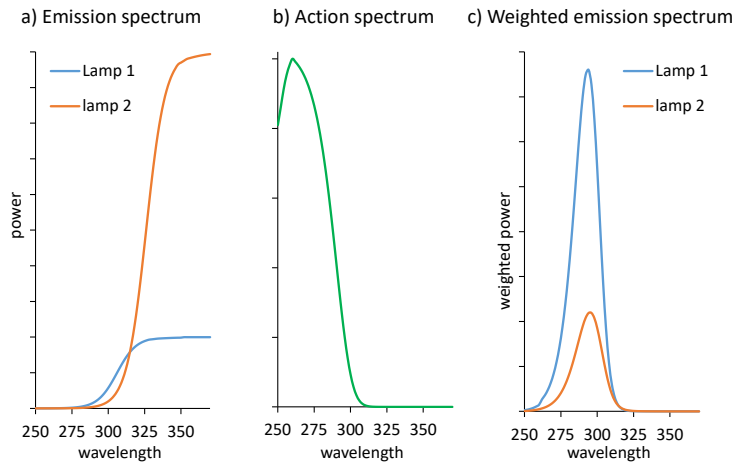
*Figure 1: Chemical structure of a) pyrimidine dimers (64PP: pyrimidine (6-4) pyrimidone photoproducts, CPD: cyclobutane pyrimidine dimers, b) various DNA bases oxidation products (8-oxoGua: 8-oxo-7,8-dihydroguanine, FapyGua: 2,6-diamino-4-hydroxy-5-formamidopyrimidine), Thy Gly: thymine glycols, 5-OHCyt: 5-hydroxycytosine)*

## 2.2. Modulation of DNA repair by UV radiation

DNA repair capacities of cells depend on numerous parameters such as the cell type or the cell cycle. In the case of UV effects, the influence of the wavelength must also be taken into account. Repair, which involves mostly nucleotide excision repair (NER) for pyrimidine dimers and base excision repair (BER) for oxidized bases and strand breaks, is modulated by UV. On the one hand, induction of the expression of NER proteins has been shown to increase the repair capacities of cells as the result of exposure to UVB. This is mostly observed upon chronic exposure [37-39] and is explained by overexpression of repair proteins mediated by various factors [40, 41] such as Interleukin-12 [42], E2F1[43],  $\alpha$ -MSH [44], the microRNA MiR-23a [45] or IGF-1 [46]. On the other hand, exposure to UVA leads to a decrease in the rate of repair of pyrimidine dimers induced by a subsequent UVB irradiation, as first observed in keratinocytes [47] and recently extended to melanocytes [48]. This phenomenon could be at least partly explained by the oxidation of DNA repair proteins [49-51]. Other explanations like the modulation of signalling pathways remain to be investigated. It may be added that other aspects of the DNA damage response are differentially modulated by UVB and UVA. Apoptosis has also been shown to be differently induced by different UV ranges [52].

### 3. The action spectrum approach

As illustrated above for DNA damage, the extent of a photobiological response strongly depends on the emission spectrum of the UV source. In order to consider this fact, photobiologists often use action spectra that report the extent of the studied response after monochromatic exposures. The results are usually normalized to the largest value set to 1. Action spectra are available for numerous processes. In the field of genotoxicity, spectra for the induction of pyrimidine dimers have been determined both in cultured cells [53] and in skin [54]. Other works simultaneously determined action spectra for both pyrimidine dimers and oxidatively generated damage in cultured cells [25, 33]. Action spectra have also been determined for the major UV-induced biological effects including skin cancer in mice [55], erythema [56] and immunosuppression [57]. Action spectra are very useful tools. They can first be used to compare different broadband sources. In this case, the emission spectrum is multiplied by the action spectrum of interest, thereby yielding a “weighted emission spectrum” (Fig. 2). For example, the erythematous weighted emission spectrum is often used to convert physical doses into more biologically relevant units, which are fractions or multiples of erythematous doses. As early as 1974, the spectrum of sunlight weighted for the induction of DNA damage allowed showing the major role of pyrimidine dimers in the induction of skin cancer [58]. The same approach further allowed predicting that UVA is the portion of sunlight playing the most important role in immunosuppression [57].



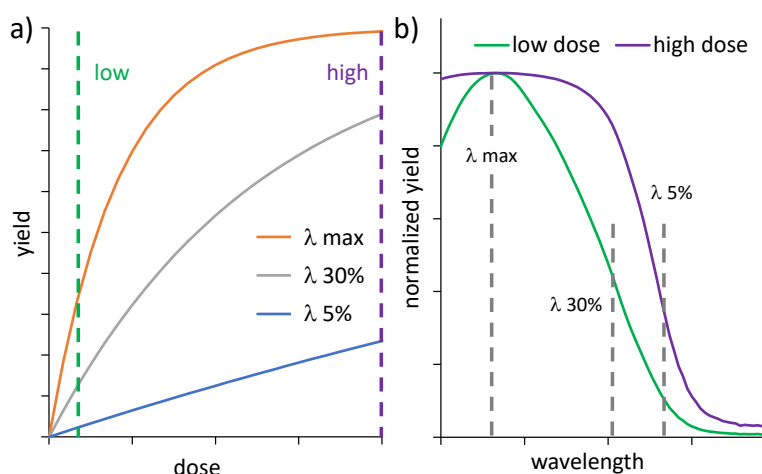
**Figure 2: Calculation of weighted emission spectra for the induction of DNA damage.** In this example, two lamps are compared, with lamp 1 emitting in both the UVB and UVA ranges being five times less powerful than lamp 2 that emits only UVA radiation. By using the Setlow's action spectrum for induction of CPDs [58], the weighted emission spectra show that lamp 1, richer in wavelengths close to the maximum of the action spectrum, it is actually more efficient than the more intense lamp 2. This illustrates how a minor proportion of UVB in a UVA source can affect biological responses.

A second interest of action spectra is the identification of the cellular chromophores involved in photobiological responses. Indeed, the wavelength corresponding to the maximal amplitude of the studied effect is a clue for to identification of the molecule at the origin of the studied phenomenon. This approach has for example provided major information in the field of photosynthesis. In mammalian cells, a nice example is the work of Coohill and co-workers [59] who used action spectra to show that DNA damage produced by direct absorption was the cause of lethality and mutagenesis induced in the UVB range. Other indirect most likely photosensitized phenomena were involved in the UVA range. The major role played by UVB-induced DNA damage in skin cancer was also supported by the analysis of the action spectrum obtained in a mice study [55]. Interestingly, this action spectrum also showed that skin was strongly protective against high-energy photons with a wavelength below 290 nm. A further illustration of the interest of action spectra in the understanding of photobiological processes is the role of DNA photoproducts in immunosuppression. This link had been suggested by experiments involving topical application of repair enzymes [60] and was reinforced by the observation of a similarity between the wavelength-dependence of the induction of DNA photoproducts and the inflammatory cytokine TNF- $\alpha$  [61]. Similar comparisons of cellular responses and DNA damage formation strongly suggested the involvement of CPD in the induction of erythema [54] and pigmentation [62].



#### 4. Pitfalls in the determination of action spectra

Determination of accurate action spectra relies on several conditions. A first point is that action spectra must be determined at a dose range where the studied phenomenon is induced in a linear dependence. Figure 3 shows how an action spectrum can be “flattened” when determined in a dose range where the induction of the studied response reaches saturation. Any conclusion made on these bases can thus be misleading since the ratio between the efficiencies of different wavelengths is wrong. This is particularly important in laboratory experiments where high intensity sources can be used. Although biologically relevant doses are rarely high enough to reach such saturation conditions, the issue has been raised for the modelling of the production of vitamin D since some of its metabolites are degraded by overexposure to UVB [63]. It may be stressed that similar considerations should be made for processes induced with a threshold effect or with bell-shaped dose-response curve like immunosuppression [64].



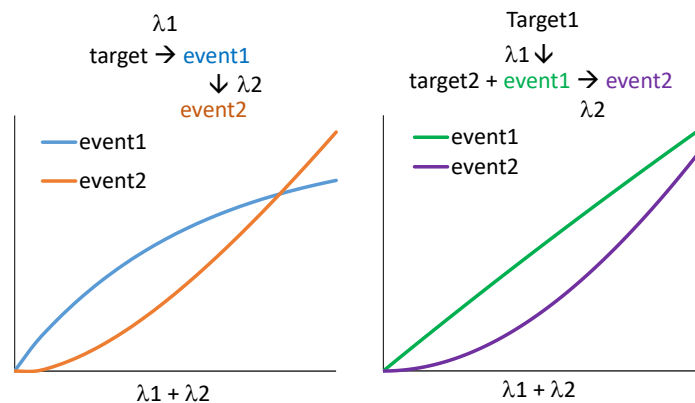
**Figure 3: Impact of linearity on the action spectra.** a) Yield of a photobiological process is determined at three wavelengths for increasing doses. The wavelengths correspond either to the maximum efficiency or to 30 or 5% efficiency, respectively. b) The normalized action spectrum for this response is determined at both low and high doses.

Another point raised is that the studied response should follow the reciprocity rule (Bunsen–Roscoe law). This means that the extent of the studied effect should depend only on the applied dose, irrespectively from the irradiation pattern. Unfortunately, this is not always the case. This has been observed for UVA-induced oxidative stress in human cells [65]. More importantly for human health, a significant effect of the fluence has been reported for the induction of skin cancer, with a higher efficiency at low rates [66, 67]. Similar observations were made for the efficiency of UVB phototherapy [68, 69]. Evidence was also provided for a strong influence of the fluence rate on the induction of oxidative stress in human cells [70]. Similar conclusions were made based on dose

fractionation experiments [65]. The role of fluence rate has thus to be kept in mind when determining and interpreting action spectra.

## 5. Non additive effects

The main limitation to the action spectrum approach is that it cannot be applied to photobiological processes induced or modulated by consecutive exposure to two (or more) different wavelength ranges. Two main situations can be encountered. The first one is the induction of a photobiological response at a first wavelength followed by a subsequent modulation in a second wavelength range (Fig. 4a). Another possibility is the onset of a response in a portion of the UV spectrum that affects the consequences of the exposure to a second wavelength range (Fig. 4b). In both cases, the correct photobiological consequences cannot be predicted or described by the action spectra because only the effects of individual wavelengths are considered in this approach. Impact of non-additivity on photobiological responses is expected for example in bacteria exposed to sunlight where UVA and visible light-drive repair by photolyases of DNA damage induced by UVB [71, 72]. Examples can also be found in plants. Photomorphogenesis was found to be activated and inhibited at two different wavelengths, which was taken into account in the determination of the action spectra [73]. Another example in plants is the variation in the concentration of various metabolites with respect to UVB to UVA ratio of the light [74, 75]. The results presented below show that similar phenomena take place in mammalian cells and in humans, in particular regarding DNA damage and oxidative stress.

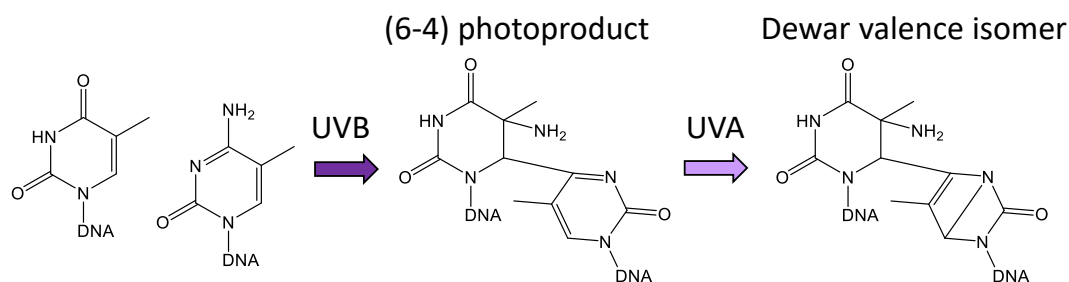


**Figure 4: Photobiological processes induced by two wavelengths.** A) Photons at a first wavelength  $\lambda_1$  hit target1 and trigger event1. The latter makes possible event2 under the influence of a second wavelength  $\lambda_2$ . As the dose increases, event2 accumulates and event1 reaches a plateau. In the absence of  $\lambda_2$ , only event1 takes place. When exposure is limited to  $\lambda_2$ , nothing happens. B) Action of  $\lambda_1$  on target1 leads to induction of event1. Combined action of event1 and a second wavelength  $\lambda_2$  on target2 induces event2. As the dose increases, the level of event1 increases and induction of

*event2 is more and more efficient. In the absence of  $\lambda 2$ , only event1 is induced while nothing happens when  $\lambda 1$  is missing.*

### 5.1. Photoisomerisation of 64PP in DNA

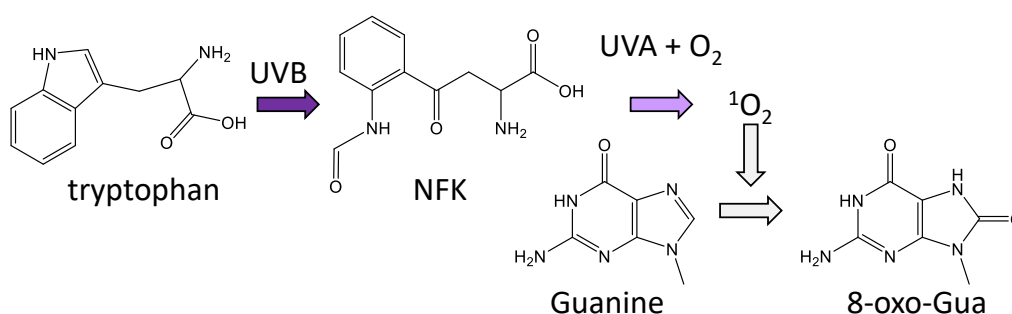
An example of the first case illustrated by figure 4a can be found in the field of genotoxicity. As explained in paragraph 2, 64PP are frequent UVB-induced DNA photoproducts. One of their major chemical properties is their ability to be converted into a Dewar valence isomer (Dewar). Dewar arise from the intramolecular electro-cyclization of the pyrimidone ring of 64PP, which exhibits a strong absorption around 320 nm [76]. Dewar can be produced by UVB irradiation of pure 64PP in solution. However, the level of Dewar is very low in cells and skin exposed to biologically relevant doses of UVB. As a matter of fact, UVB is absorbed mostly by normal bases and not by the minute amounts of 64PP. Dewar is also basically absent in the DNA of cells exposed to UVA. This UV range does not induce 64PP that necessary precursors for Dewar [18, 30, 77]. A theoretical action spectrum, calculated on the basis of available absorption coefficients and quantum yields, exhibited a maximal yield of Dewar that was 4 orders of magnitude lower than that of CPD and 64PP [78]. This would suggest that Dewar is a very minor DNA lesion. However, Dewar are observed in large amounts when cells and skin are exposed to a combination of UVB and UVA such as natural [79-81] or simulated sunlight [18, 77, 82]. In this case, UVB induces 64PP, which then efficiently absorb UVA photons and undergo photoisomerisation (Fig. 5). Dewar appears thus as an important class of DNA damage to consider [83].



**Figure 5: Formation of Dewar valence isomer in DNA upon combined exposure to UVB and UVA.** 64PP is produced in large amounts in the DNA of cells exposed to UVB. Because of the overwhelming presence of normal bases that absorb the vast majority of UVB, no photoisomerisation takes place. In contrast, UVA is very weakly absorbed by DNA but more efficiently by 64PP. UVA can thus induce the photoisomerisation of UVB-induced 64PP. It should be stressed that UVA alone does not induce 64PP.

## 5.2. Complex photoreactions leading to oxidative stress

Photoinduced processes involving photons at two wavelengths may also involve photosensitization reactions. In this case, the dose-dependence is the type described in figure 4b, with event1 being the formation of an endogenous photosensitizer. For example, recent results from test tube experiments suggested that DNA damage such as 64PP [84, 85], once formed in DNA, could behave as internal photosensitizers for the formation of CPD or oxidized bases. Although this reaction was found to be poorly efficient [86], it is an example where UVB is required for inducing 64PP and UVA to perform the photosensitization step. The same scheme applies to other biomolecules that can be converted into a photosensitizer in one wavelength range and then induce oxidative stress in another. This is the case of pterin derivatives [87, 88 ] and of some tryptophan photoproducts [89-91]. These compounds are produced by UVB radiation and induce photosensitized-oxidative stress upon exposure to UVA (Fig. 6). Formation of further cellular damage such as the oxidized DNA bases can be anticipated. In a similar way, UVA-induced lipofuscin was reported to photosensitize keratinocytes to visible light [92]. Other combination effects are possible. The recent report of a protective effect of antioxidants against the formation of CPD suggests that UVA-induced oxidized products could behave as DNA sensitizers [93].

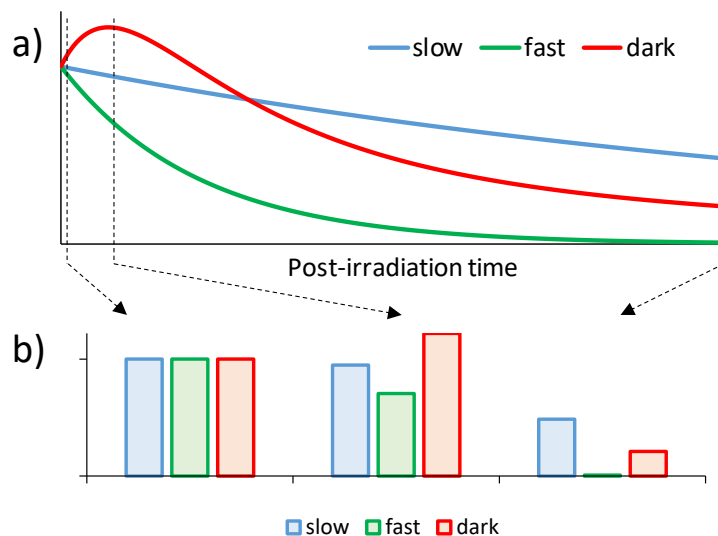


**Figure 6: UVB-induced formation of a UVA-photosensitizer.** The represented cascade of events shows the formation of *N*-formylkynurenine (NFK) from tryptophan. NFK can then absorb UVA and produce singlet oxygen, which is at the origin of the formation of 8-oxo-Gua in DNA

## 6. Time as a major parameter in predicting photobiological events

Time plays a major role in the onset of biological effects. Differences are often observed between the temporal evolution of different photobiological responses to UV radiation after exposure. Cascades of events may take place in the minutes and hours after irradiation. Therefore, simultaneous events may not have the same intensity at different times following exposure. Figure 7 illustrates the examples of three different photobiological responses exhibiting different temporal evolution. One decreases rapidly, the other much more slowly while the last one undergoes a phase of “dark induction” after the end of the irradiation. The relative induction of the three studied processes is

strongly time dependent. Such considerations thus have important consequences in the understanding of biological processes and must be considered when action spectra are used to simultaneously study several endpoints. The importance of these temporal effects is illustrated below for the impact of DNA repair on mutagenesis and the role of photoinduced “dark chemistry” in the induction of cellular damage.



**Figure 7: Temporal effects on photobiological responses.** Panel a) shows the temporal evolution of three phenomena after irradiation. “Slow” and “Fast” corresponds to responses that decrease with either a slow or a fast rate, respectively. “Dark” corresponds to a process induced during as well as after irradiation and then decreases. (b) The relative inductions of the three processes are no longer the same at different times after irradiation. The reported ratio were calculated immediately after irradiation, at the maximal induction of the “dark process and at the almost complete disappearance of the “fast” response.

### 6.1. Mutagenesis and DNA repair

Unravelling the contribution of the different types of DNA lesions to mutagenesis requires taking into account their respective yield, their intrinsic mutagenic properties and their persistence in the genome. Indeed, only damage present when cells divide can be converted into mutations. A half-division time of 24 h for keratinocytes *in vitro* and even longer in skin provides an estimation of the lifetime necessary for a DNA damage to be mutagenic. In terms of mutation, solar mutagenesis has been known for years to be dominated by C to T transitions at TC sites and CC to TT tandem mutations [15, 94-97]. These results have been strengthened by last generation sequencing techniques applied to thousands of human tumours in order to define specific mutational signatures [98-100]. These mutations arise from the formation of pyrimidine dimers. Experiments involving overexpression of specific repair enzymes showed that mutations induced by UVB result more from CPD than from

64PP [101, 102]. This could be surprising because *in vitro* shuttle vector experiments have shown that the intrinsic mutational properties of 64PP are much larger than those of CPD [103-105]. This apparent contradiction can be explained by the difference in the repair rates. 64PP are removed from cells within a few hours by nucleotide excision repair while CPD are still present 24 or 48 h after irradiation [106-108]. Faster repair of CT CPD compared to TC CPD [109] also explains why mutation at CT are rare events in UV-irradiated cells. In contrast to that of pyrimidine dimers, the signature associated with oxidatively generated damage is rarely encountered in skin cancers, as shown by the absence of the corresponding mutational signature in melanoma [98, 100]. Again, fast repair is a likely explanation to this result, in addition to the fact that oxidized bases and strand breaks are produced with a much lower efficiency than CPD [25, 77]. Because of its efficient repair, the half-life time of 8-oxoGua in cells is in the range of 2-3 hours [110-112]. The repair of single-strand breaks is even faster. Altogether, UV-mutagenesis is a nice example of a situation where action spectra determined immediately after exposure are not relevant. They may yet be useful for the determination of DNA damaging photochemical pathways or for the study of processes where DNA damage is an early sensor.

## 6.2. Post-irradiation “dark chemistry”

The literature also reports that some responses increase not only during but also after irradiation. An example can be found again in the field of DNA damage. CPD were found to be produced within the first few hours following UV irradiation of melanocytes [113]. It was proposed that melanin derivatives were oxidized into endoperoxides that decompose into activated species undergoing energy transfer to DNA bases. Other mechanisms could be involved since similar observations were also made in keratinocytes [93]. In both cell types, involvement of oxidative stress is strongly suggested by the inhibition of this process by antioxidants [93, 113]. Oxidative stress is also possibly induced in a post-irradiation period as shown by the sustained production of reactive oxygen species (ROS) minutes and hours after the end of irradiation [114-116]. Production of ROS can also indirectly induce dark chemistry through the formation of intermediate oxidizing species. One example is the formation of protein peroxides [117, 118]. It was also reported that peroxidised proteins are able to induce oxidative damage when mixed with DNA [119, 120]. One can thus foresee that oxidized proteins like histones could be the vector of oxidation reactions in the DNA of irradiated cells after the end of the exposure. This possible role of post-irradiation induced oxidative stress is strengthened by the observation of the induction of persistent genomic instability by UVA [121].

## **7. Combined spectral and temporal effects**

We have seen so far that both non-additive effects of different wavelength bands and temporal effects could limit the accuracy of predictions based on action spectra. Both phenomena may actually take place simultaneously and further complicate the prediction of photobiological responses. A nice illustration is related to DNA repair. As reminded in paragraph 2, UVB and UVA exhibit opposite effects on the efficiency of DNA repair. Therefore, it is expected that the rate of repair of DNA damage will depend on the spectral composition of the incident radiations. UVB-rich sources would accelerate repair while UVA-rich ones would have the opposite effect. Consequently, even if the level of damage is similar immediately after irradiation, the amount of residual damage, which is responsible for mutagenesis, will depend on the UVB to UVA ratio of the source. This effect has been reported for the extent of UV-induced apoptosis that depends on the UVA/UVB ratio [122]. Photoinduced immunosuppression is an even more complicated case. Indeed, it has been shown to be induced faster by UVB than by UVA [123, 124], strongly suggesting a major role of the spectral composition on the extent of the response. It is thus expected that short-term immunosuppression results mostly from UVB while UVA plays a role at longer times. In addition, the immunosuppressive effect of UVB is counteracted by UVA [125, 126]. In this case, both temporal and spectral effects are critically linked and make the use of action spectra almost pointless.

## **8. Conclusion**

The above discussion shows that, although powerful tools, action spectra may suffer from limitations associated with possible lack of linearity of the studied response in the considered dose range, non-additive effects between effects of radiations of different wavelengths, and modulation over time of the response induced immediately after irradiation. In order to avoid misleading conclusions, some guidelines can be proposed:

- When wavelength effects are not central to an experiment, use of a solar simulator should be preferred to pure UVB or UVA emitting lamps;
- When studies are performed with either UVB or UVA, a control with both wavelength ranges should be included;
- Action spectra should always be determined following exposure to several doses to check the linearity of the induction of the studied response;
- The time-dependence of the response should be studied before determination of an action spectrum.

Most photobiological studies, especially those related to cutaneous and systemic effects, are focused on the UV range of sunlight because of its strong biological impact. Evidence that lower energy

photons also exhibit biological effects are however accumulating. Infrared light is known for years to have both beneficial and deleterious effects on skin [127-129]. Blue light is also active, as shown by the formation of oxidatively generated DNA bases [33] and more recently by the induction of melanogenesis [130]. A higher level of complexity may combine the impact of circadian rhythm, mostly driven by blue light, with cellular responses such as repair of UVB-induced DNA damage [131, 132]. The general conclusion of this survey is that novel approaches based on innovative mathematical modelling of biological responses to light are necessary for a better understating and prediction of effects of light on living systems. Interestingly, the field of chemical toxicology provides numerous evidence for the lack of additivity of the biological responses to the components of mixtures, resulting in either synergistic or inhibitory effects [133-140]. Interesting mathematical tools have been developed to provide evidence for non-additivity effects [141]. Photobiology could benefit from using similar approaches.

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