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Review article

Recent advances in confocal microscopy for studying drug delivery to the eye: Concepts and pharmaceutical applications

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ABSTRACT

Since its seminal introduction 50 years ago, confocal microscopy has been applied in numerous fields in life sciences. This review presents the different key elements of confocal microscopes, in particular scanning techniques, light sources and especially laser sources are described in this review. Furthermore, an overview of the different image processing systems coupled with confocal microscopy is provided. The chapter closes with the applications of confocal microscopy in drug delivery to the eye.

1. History of confocal microscopy

In the 17th century, the Dutch Leeuwenhoek invented a single lens microscope that enabled the observation of many cells and tissues in the body and specially the corneal epithelium and the lenticular fibers of the ocular lens [1]. The conventional optical microscope was a milestone for biological medical sciences which permitted many important discoveries. However, the desire of scientists to look in more and more details into biological materials stumbled against several limitations of the instrument. The optical microscope, for instance, does not allow observing details of living tissues. The invention of the confocal microscope enabled to overcome this limitation. The main stages of the development of confocal microscopy designed for the observation of the eye are summarized in Table 1. Helmholtz, in 1850, designed an ophthalmoscope to permit the clinician the examination of the living retina [1]. The next major advance was the development of original scanning devices capable to enlarge the field of view of microscopes. Indeed, in 1884, a young student in Berlin, Nipkow figured out how to scan an image in a raster pattern using a spinning opaque wheel perforated by a series of holes [2]. This simple scanning device was the precursor of television [3,4]. In the middle of the 20th century, technological advances in optics and electronics that had been brewing for several decades contributed to the successful development of confocal microscopy and led to the design of the first confocal microscope [5,6]. As a young postdoctoral fellow at Harvard University, Marvin Minsky applied for a patent in 1957 regarding a microscope that used a stage-scanning confocal optical system [7]. The microscope was developed for studying neural networks in the living brain. Some years later, in 1968, Petran and coworkers in Prague designed a tandem scanning confocal microscope (TSCM) using a Nipkow disk [8]. The first confocal laser scanning microscope (CLSM) was built in 1971 by Davidovits and Egger [9].

A slit scanning confocal microscope was designed by Koester in 1980 and found recent application for the observation of the cornea [10,11]. More recently, Gordon Kino at Stanford University has designed a modified TSCM with a special Nipkow disk [12]. There are three main reasons which permit the confocal microscopes to become commercially available in the 1980s: (i) the advent of less expensive personal computers; (ii) PC-based image processing software; and (iii) small air-cooled lasers [5,13].

2. Principle of confocal microscopy

In his memoir on inventing the confocal scanning microscope, Minsky [7] reported that the main problem in standard microscopes was the scattering of light resulting in blurred images. He stated that [7] "One day it occurred to me that the way to avoid all that scattered light was to never allow any unnecessary light to enter in the specimen". He invented the so-called point illumination (Fig. 1).

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Table 1
Historical overview of the main milestones in the development of confocal ophthalmoscopy.

Year	Authors	Contributions	Reference
1850	Helmholtz	Invention of the ophthalmoscope	[14]
1873	Abbe	Foundation for modern light microscopy	[15,16]
1884	Nipkow	Image scanning in a raster pattern	[2]
1957	Minsky	US patent for a new confocal microscopy apparatus	[7,17]
1968	Petran et al.	Tandem scanning confocal microscope (TSCM)	[8]
1971	Davidovits and Egger	Laser illumination: confocal laser scanning microscope (CLSM)	[9]
1977	Sheppard et al.	Theory of confocal optic and laser scanning	[18]
1979	Brakenhoff et al.	Specimen scan	[19]
1980	Koester	Slit scanning confocal microscope	[10]
1983	Cox et al.	Digital recording	[20]
1984	Wilson and Sheppard	Extended depth of field	[21]
1985	Carlsson et al.	Stacks of confocal images	[22]
1985	Wijnaendts van Resandt	X-z view	[23]
1986	Lemp et al.	TSCM used to observed the cornea	[24]
1986	Susuki-Horikawa	Video rate laser scan	[25]
1987	Xiao and Kino	Modified TSCM (Kino type monoscanning confocal microscope)	[12]
1987	Amos et al.	Stereo imaging	[13]
1988	Masters	CLSM used to observed the cornea	[26]

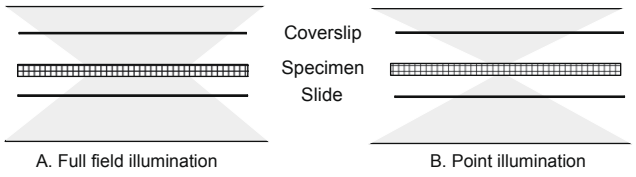

Fig. 1. Comparison of specimen illumination in conventional fluorescence microscope (A) and confocal microscope (B).

The conventional microscope has a wide-field illumination (Köhler illumination); thus, the whole depth of the specimen is continuously illuminated. As a consequence, the out-of-focus signals as well as the focal plane of interest are detected. In confocal microscope, a single diffraction-limited point at the focal plane is illuminated. The volume occupied by the specimen between the coverslip and the microscope slide is marked out in squares, and the cone of illuminating light is shown shaded. For simplicity, refraction effects are not shown [27].

Point illumination is achieved by a special optical arrangement in which the condenser and objective lenses have the same focal point, hence the designation confocal which is a contraction of conjugate focal plane [28,29]. This arrangement enables the optimal illumination and detection of the same point [30–32]. This focusing on a small area limits the total amount of scatter, but it does not prevent light from being reflected and scattered by illuminated tissue lying above and below the spot of interest [33,34].

By means of a mask containing a pinhole aperture, Minsky succeeded in filtering out most of the light emanating from tissue outside the spot [7].

Fig. 2 shows the optical principle (confocal arrangement and pinhole) of confocal microscopy used for the observation of the cornea.

Confocal optics is designed to restrict the final image visualized to light coming only from a focal plane. This is achieved by focusing the light source within a small area of the tissue and focusing the condenser on that same area. Thus, both the condenser and the objective lenses have the same focal distance (f_1). When the object (cornea) is simultaneously in the focal plane of the light source and detector (in focus plane), the reflected light is focused on the pinhole (dotted line). Other parts of the object that are out of the focal plane are stopped at the pinhole and are not imaged by the detector (solid line).

Owing to the optical arrangement of lenses combined with pinholes, confocal microscopy has an improved resolution compared

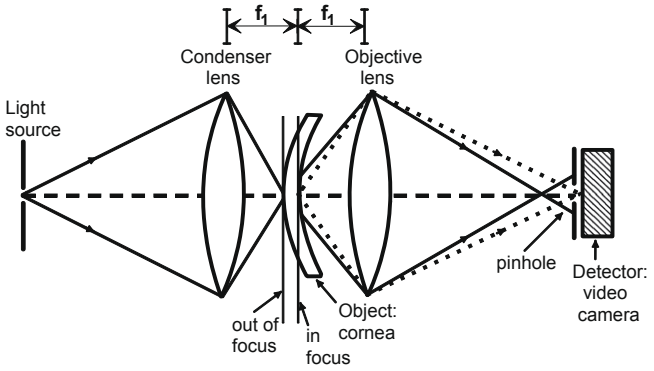

Fig. 2. Concept of optical sectioning in confocal microscopy. Adapted from [35].

to standard light microscopes, but this improvement is made at the expense of field of view according to the principle of Lukosz [36–38]. The reconstruction of a whole image is possible by scanning the object.

3. Scanning techniques

The use of a point source/detector in the confocal optical design trades field of view for enhanced resolution [39,40]. Therefore, to recover a full field of view, a scanning system is necessary. Different methods of scanning may be employed to achieve confocal imaging. As a result, three major scanning designs have emerged to date [41]:

- stage scanning: a stage is moved laterally under a stationary beam of light,
- beam scanning: a beam of light is scanned over a stationary stage,
- tandem scanning: both stage and light source are stationary.

Fig. 3 gives schematically the design of the three main types of scanning devices.

(A) Stage scanning: the image is formed by moving the specimen through a stationary spot of light focused in the object plane. (B) Beam scanning: the image is formed by moving a spot of laser light across the stationary object plane in a rectangular raster pattern. (C) Tandem scanning: the image is formed by simultaneous illumination by multiple spots focused in the object plane. Each

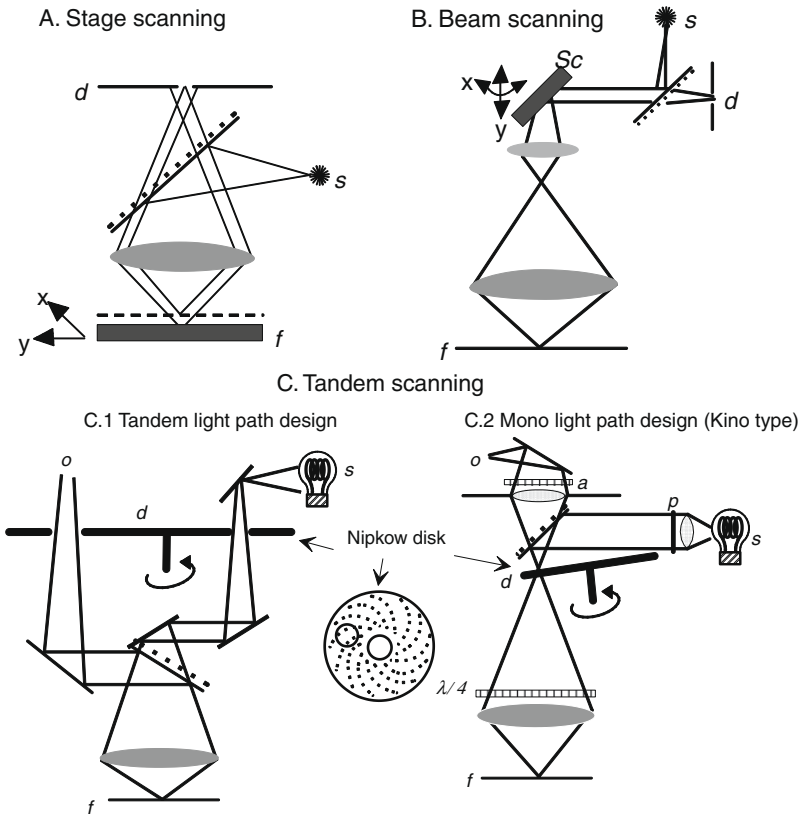

Fig. 3. Schematic drawings of the three types of scanning devices. Adapted from [42].

spot is the image of a pinhole aperture in the Nipkow disk. (C1) Tandem light path scanning design, (C2) mono light path scanning design (Kino type). *f*: focal plane in the object; *s*: light source; *d*: detector aperture; *o*: ocular; *a*: analyser; *p*: polarizer; *Sc*: laser scanner (galvanometer mirrors); $\lambda/4$: quarter wave plate. The detector aperture is placed in a conjugate image plane in all cases.

The stage-scanning type has two advantages. Firstly, a much larger field of view than the one obtained with high-resolution objective, and secondly, minor optical aberrations from objective and condenser, resulting from a constant axial illumination due to the stationary optical arrangement [9,18,20,21,23,43–45]. However, there are two limitations with this scanning type: the relatively low scanning speed and the necessity to firmly fix the specimen to the stage in order to avoid any shift that could produce a distorted image. This technical requirement prevents *in vivo* observation [46].

The beam scanning type employs usually rapidly scanning mirrors or acoustic-optical deflectors to scan a laser beam across the specimen [47]. Scanning mirrors are highly reflective galvanometer mirrors that produce the vertical and horizontal patterns of the raster scan. Mechanical limitations of the galvanometers restrict the speed at which a single frame can be scanned; a full $512 \times 512 \times 8$-bit image may take as long as 10 s to acquire [48]. As a result, rapid cellular changes, such as ionic changes, cannot be imaged at high resolution. The second scanning device is the acousto-optical deflector. This instrument is an electrically controllable diffraction grating that generates the high-frequency horizontal scan of the laser beam. This light beam is diffracted vertically by acoustic waves generated by a piezoelectric transducer [49]. This scanning system has the advantage to produce images at video-rate.

This device is used in confocal laser scanning microscope (CLSM). It provides superb resolution and a faster scan rate than the stage scanning instruments [22,47,50]. Furthermore, this laser scanning device is particularly suitable for fluorescence microscopy as common laser light efficiently excite many fluorochromes used in biological applications without any out-of-focus blurring [49,51].

The third scanning device is the tandem scanning [52,53]. Its very fast scan rate allows real-time imaging. Indeed, in order to achieve this high scan, this system employs, instead of a single pinhole, thousands apertures (14,000–200,000) drilled in a ceramic–copper or glass–metal disk (Nipkow disk) in an Archimedean spiral arrangement. As the disk rotates at high rate (180–2500 rpm), each single spot scans a line and the pencil of light going through the disk covers, in succession, the whole field of view. Each hole is of order of 20–80 μm diameter [35]. The arrangement of the pinholes gives the disk a central symmetry such that each hole has an exact conjugate pair on the same diameter and at the same radial distance from the center but on the opposite side [54]. Thus, one pinhole serves for incoming illumination and its conjugate for the detection of excited light. By means of few modifications (tilting the disk, using internal light stops, polarizing the light and covering the disk with black chrome) in order to reduce internal reflectance and therefore to enhance the signal-to-noise ratio, a scanning device (scanning of Kino type) was developed which uses a single light path, one pinhole serving for both illumination and detection.

4. Light sources

Light sources used in confocal microscopes are either polychromatic (incoherent) or monochromatic (coherent) [54]. Polychromatic light sources emit white light, which contains a large range of wavelengths. Tungsten-ribbon lamp, quartz–halogen lamp, xenon, mercury, carbon and zirconium arc lamps belong to this cat-

egory [55]. The advantages of these broadband light sources are their availability, low cost (purchase and maintenance), wavelength flexibility (by using color filters) and simplicity. The polychromatic light sources are usually used in tandem scanning confocal microscopy (TSCM).

Lasers represent the second class of light sources (monochromatic light). They were first used as a light source in 1969 [56] and are now widely used in confocal microscopy, mainly in confocal laser scanning microscope (CLSM). Compared to polychromatic sources, lasers have a number of unique properties which make them an almost ideal light source for use in confocal microscopy [57]. These properties are as follows:

- high degree of monochromaticity enabling to work at a given wavelength, which proves to be very useful in fluorescence microscopy,
- small divergence and high degree of coherence making it possible to focus the light beam on a small point,
- high brightness.

Nevertheless, there is one drawback with monochromatic light: the observation is not possible in real colors. Since the discovery of laser by Maiman in 1960, a wide and still expanding variety of lasers has been developed [5]. The ophthalmology has been one of the first medical field to use laser for both diagnosis and treatment [58,59]. Excimer lasers are used as "optic scalpel" for refractive surgical procedures (correction of myopia, hyperopia and astigmatism) [60,61]. Argon laser is used for the photocoagulation to prevent retinal detachment, to treat diabetic retinopathy or to treat surgically glaucoma. Argon laser is also used for angiography. Finally, Nd-Yag laser has proved to be useful to operate secondary cataract. The wavelengths of essentially all lasers in common use are near the micrometer spectral line, ranging from 10^{-3} to 10^{3} μm, thus covering the ultraviolet, visible and infrared region of spectrum [62].

Safety considerations in the clinical use of laser in ophthalmology are important, especially if lasers are meant for a diagnostic purpose [63–65]. As laser beams are powerful, an uncontrolled use of these light sources can produce ocular damages by thermic or photochemical effects (Fig. 4) [66].

Laser beams with a wavelength near the visible can reach the retina and may cause retinal burns whereas laser beams with wavelength lower than 400 nm or upper 1.4 μm are absorbed by the anterior elements of the eye and may provoke corneal burns [67].

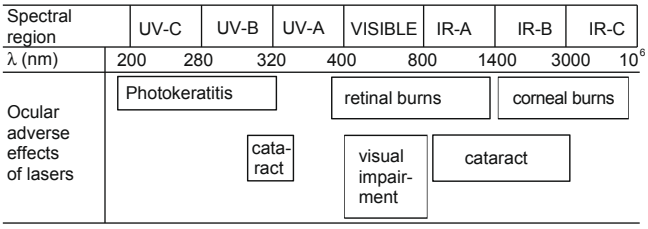

Fig. 4. Possible ocular adverse effects caused by laser light according to the wavelength [67].

By exposing rabbits or rhesus monkeys to laser radiation of various types and evaluating histologically any ocular damage, the American National Standard Institute (ANSI) established the laser-eye damage thresholds [68–70]. Lasers were classed into four groups according their potential harmfulness [57,71,72]. Table 2 gives an overview of the different laser classes and the general safety precautions to be followed while operating a laser.

It is clear that the eventual risks associated with the use of laser depend on many factors: power of laser, time of exposure and type of laser. Respecting the high safety standards, confocal microscopy can be used without danger in clinical studies of the human eye.

5. Image processing systems

Confocal microscopy benefits enormously from the advances realized in the development of computers for digital imaging processing [33,74]. Actually, most confocal microscopes use software to control the microscope during image acquisition and to further process the confocal images. Images are processed either during or after acquisition [49].

Many image processing methods are available; they mainly involve three procedures: image manipulation, image display and image analysis [75]. Among the numerous image manipulations (contrast enhancement, image alignment, smoothing), the most used image processing is the three-dimensional reconstruction [76–78]. It consists in stacking the optical sections obtained by focusing through the specimen at different levels (Fig. 5) [22,30,79–81].

A specimen (mitotic sea urchin egg) is carefully mounted to prevent the coverslip from flattening the cell (on the left of the drawing). A through-focal series of optical slices, known as a *z* series, is then taken to generate a volume data set (on the right). The addition of the different optical sections results in a three-dimensional reconstructed image.

Image analysis permits the quantitative evaluation of images [82]. Different operations can be carried out on the image such as calculation of distances, surfaces, angles and volumes. The vast amount of collected information present can be displayed in different ways: rotation of the specimen, projection on a given surface, coloring and codification of the different light intensities, dyes labelling, shading to amplify the volume of the object and finally choice of the background color or even the interactive animated 3D rotation [41]. Generally, there are three different manners to render confocal images [41]. The first method is a three-dimensional reconstruction that may be spectacular for thick specimens in the way that the image gives a good idea of the spatial form of the specimen. The second common practice is to represent the different optical slices as a *z* series montage composed of various optical sections taken at regular intervals. The third possibility to represent confocal images is the stereo pair that consists to provide two images of the specimen viewed from two different angles that give the illusion of three-dimensional (the difference between angles is approximately 6°) [49,83,84]. Red–green glasses enable to view the object three-dimensionally [82].

Table 2
Classification of lasers according their risk of use (adapted from [67,73]).

Class of laser	Characteristics	Safety precautions
Class I	Laser of very low power, no risk	No user safety rules necessary
Class II	Laser of low power, low risk	Do not purposefully stare into the laser longer than 10–20 s
Class III	Laser of medium power, moderate risk	Do not direct the laser towards an eye, only for experienced personnel, control spectator, restricted area
Class IV	Laser of high power, important risk	Skin and eye protection, controlled workplace

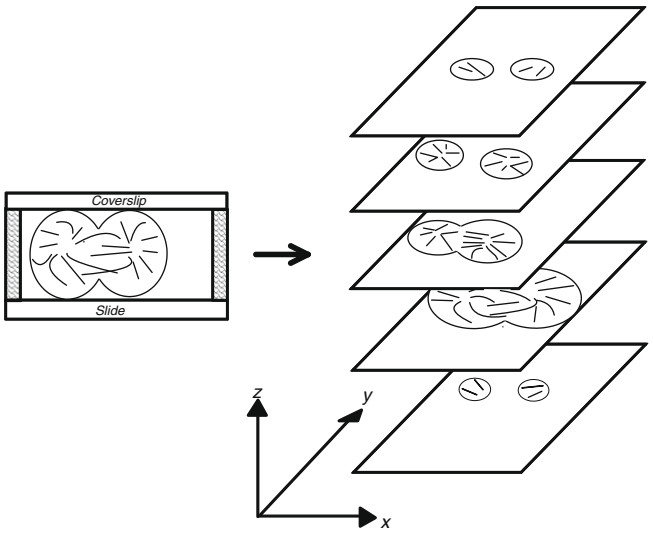

Fig. 5. Schematic demonstration of the principle of optical sectioning and three-dimensional reconstruction. Adapted from [49].

6. Application of confocal microscopy in drug delivery

Many fields in life sciences have taken advantage of the exceptional quality of images provided by confocal microscopy. For instance, in biology, mycology and pharmacology, the high resolution and improved contrast have been exploited to histologically examine tissues at cellular level [27,85–88]. However, the application of confocal microscopy to studying drug delivery is not as widespread as its application within the field of biology.

The combination of fluorescence technique with confocal microscopy provides a comprehensive tool for investigating drug delivery [89]. Confocal laser scanning microscopy (CLSM) has applied to characterize pharmaceutical systems like colloidal systems, microspheres, pellets, tablets, film coatings and hydrophilic matrices [90]. For instance, Fu et al. [91] used pH-sensitive fluorescent dyes to image and measure the microenvironmental pH during the degradation of poly(lactic-co-glycolic acid) (PLGA) microspheres, a controlled-release drug delivery system. CLSM has also been used recently to study bioadhesive dosage forms, in particular to monitor the duration of polymer retention on a mucosal membrane [92]. Additionally, the improved resolution of CLSM enabled to study the structure and the time evolution of transient gels formed in colloid–polymer mixtures and has demonstrated that large differences can exist in the local structure within a single system; this type of study would be extremely difficult using conventional light or fluorescence microscopy techniques [90].

Contrary to conventional light microscopy, CLSM enables direct examination of viable tissues at high resolution (0.2 μm) without requiring fixation and sectioning, which can lead to artefacts. The application of CLSM for studying transdermal drug delivery has been comprehensively reviewed elsewhere [93]. For instance, using different fluorescence probe that can be excited with an appropriate laser at a wavelength that does not interfere with the skin's autofluorescence, it is possible to visualize the penetration pathway of dermatological formulations. Thus, CLSM may contribute to the determination of the mechanisms of various skin penetration enhancement strategies.

Ophthalmology is one of the fields that take the most advantage of the potential for high-resolution imaging, non-invasive optical sectioning and three-dimensional reconstruction of confocal microscopy combined with sensitive, selective and versatile fluorescent probes [94]. Confocal microscopy is currently applied to clinically examine the anatomy and pathology of ocular structures [95,96]. CLSM is also a useful tool in the formulation of

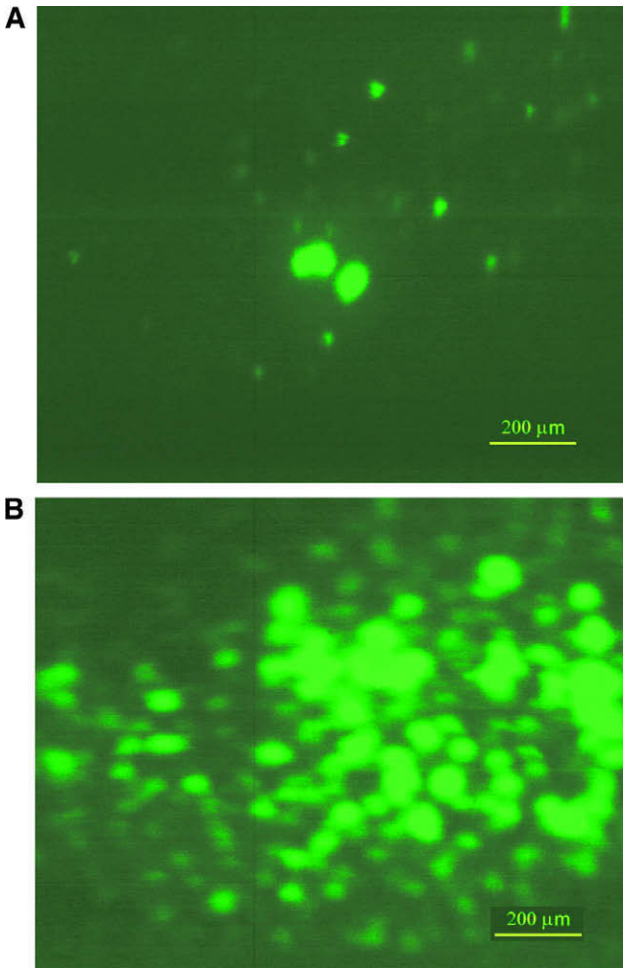

Fig. 6. Comparison of the corneal irritation on rabbit corneas after the iterative instillation during 3 days of two absorption enhancers: DMSO (A) and fusidate (B), both at 1%. The bright areas correspond to damaged zones, whose surface represents 2.5% for the DMSO and 38.2% for the fusidate. The scale bar is 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

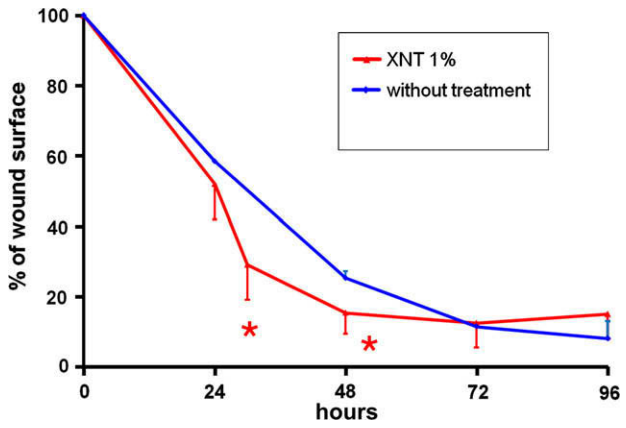

Fig. 7. Evolution of the rabbit corneal wound healing after circular mechanical wound of 6 mm diameter ($n = 8$, mean + SD). The effects on wound healing of a hydrogel (1% xanthan gel – XNT 1%) are compared with the absence of treatment (without treatment). The percentage of fluorescent corneal surface is assessed by confocal microscopy. $^{*}p < 0.05$, Student's t test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 3
Applications of confocal microscopy for studying drug delivery.

Field	Applications of confocal microscopy	Reference
Dermatology	Determination of the penetration across the skin of numerous drugs, study of the follicular route	[110]
	Study of the large hydrophilic compounds transport across the skin by opening the tight junctions with the aid of permeation enhancers	[111]
	Characterization of permeation enhancers in transdermal patches	[112]
Nasal formulations	Visualization and quantification of the water ingress into lyophilized nasal formulations	[113]
Microparticulate formulations	Study of the release profile of model drug from PLGA microspheres, showing that the rate-limiting step is the swelling rate of the polymer matrix	[114]
	Measurement and mapping of pH modification during the hydratation of pellets containing pH-sensitive fluorophore and weak acids intended to promote drug solubility/stability	[115]
Ophthalmology	Study of the corneal penetration route of labelled peptides in the presence of permeation enhancers	[116,117]

ophthalmic dosage forms: it enables to assess *in vivo* the ocular tolerance of ophthalmic drugs. Tolerance is one of the four requirements of ophthalmic drugs besides stability, sterility and efficacy [97]. Obviously, any lesion causing a disruption of the corneal anatomical features (neovascularization, pigmentation, swelling or scarring) will alter corneal transparency and consequently impair the vision [98]. Furthermore, as the corneal epithelium is very richly innervated by sensory nerve endings, corneal damage can be, therefore, very painful [99]. The *in vivo* technique devised 50 years ago to assess ocular tolerance of chemicals and called Draize test [100–102] relies on macroscopic observation and scoring of ocular changes (degree or extent of opacity on the cornea; redness on the iris; chemosis, i.e. swelling, redness and discharge on the conjunctiva) consecutive to the instillation of chemicals onto the eye. This test is sufficient to characterize the potential toxicity of chemicals that may accidentally splash on the ocular surface. But, it is not well adapted for the evaluation of ophthalmic preparations because of its lack of objectivity, repeatability and predictability of the human responses [103–105]. On the contrary, CLSM enables to observe *in vivo* corneal injury labelled by fluorescein. For instance, the ocular tolerance of excipients like surfactants, permeation enhancers (Fig. 6) or surface-active polymers, used in the formulation of eye drops, as well as active ingredients or prodrugs have been evaluate directly *in vivo* [106–109]. This method is simple to perform, give consistent objective results and is sensitive enough to discriminate between low irritancies. It can also be used to follow-up the wound-healing process after ocular surgery and to measure the effect of drugs in promoting wound healing (Fig. 7). Table 3 summarizes some examples of the application of confocal microscopy for studying drug delivery.

7. Conclusions

Seldom, the introduction of a new optical instrument has generated such a great excitement among scientists in life sciences as the confocal microscope. With this new microscope, one can slice thin optical sections out of thick specimens with improved resolution and enhanced contrast; one can penetrate deep into light scattering tissues and get impressive three-dimensional views. Confocal microscopy has been used in many scientific fields, and it will doubtless create exciting new challenges and possibilities. The potential of the confocal technology is enormous especially in both the clinic and the basic science research laboratory where clinician and scientist can take advantage from the non-invasive ability of confocal microscopy to observe *in situ* the anatomy and physiology of living tissues as well as to characterize pharmaceutical systems. As a result, confocal microscopy has emerged as a powerful tool in drug delivery.

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