QUANTITATIVE 3D-MICROSCOPY FOR PHOTODYNAMIC THERAPY OF CANCER CELLS

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ABSTRACT

Localization and eradication of tumor cells without affecting or damaging normal tissue is a challenging target in today's cancer therapy. This objective could soon be viable through the use of Photodynamic Therapy (PDT), a promising tumor treatment modality based on the combined application of laser light in conjunction with a photosensitizing agent. Appropriate sensitizer show a preferential accumulation in tumor cells where they become cytotoxic by producing singlett oxygen after illumination with light of a specific wavelength.
Confocal laser scanning microscopy offers an efficient way to acquire 3D-image data sets of tumor cells or tissue for postliminary quantitative analysis. This paper outlines the various impeding effects which need to be accounted for if photosensitizer dye concentrations are estimated from stacks of CLSM data. To address the specific needs for quantitative 3D-microscopic investigations efficient tools and procedures for image enhancement and restoration, interactive identification, segmentation and 3D-analysis of cell structures or cell colonies are a prerequisite.

1. INTRODUCTION

The more traditional forms of cancer treatment like surgery, irradiation by X-rays or chemotherapy are most widely applied and have proved to be successful. These treatment modalities present, however, disadvantages such as invasiveness, considerable side effects, often unspecific targeting of the tumor tissue and the inability to act down to the single cell level.

Localization and eradication of tumor cells without affecting or damaging normal tissue is a challenging goal in today's cancer therapy. This objective may soon become a reality thanks to a promising cancer treatment modality named Photodynamic Therapy (PDT) [1, 5] which has the potential to selectively destroy benign as well as malignant proliverative cells.

In 1994 the US Federal Drug Administration (FDA) approved the use of PDT as a valid tumor therapy.

PDT presupposes the preferential absorption of photosensitive agents, such as hematoporphyrin derivatives (phtalocyanines and chlorines among others) by cancerous cells. The accumulated sensitizer is non-toxic until

exposed to light illumination of a wavelength between 600 and 700nm (therapeutical wavelength). Photoactivation induces a toxic effect in the tumor cells by producing singlet oxygen ($_1O^2$) and/or free radicals in the cytoplasm. This reaction which takes place mainly in the dark after light exposure, affects essential organelles in their function (cell membrane, mitochondria) and leads to tumor cell necrosis.

In addition to the therapeutical application and in contrast to the traditional cancer treatment modalities photosensitizers are very well suited for diagnostic purposes. Laser light irradiation of shorter wavelength (488nm or 514nm) induces a strong fluorescence which is utilized to localize and quantify the extend of tumor cells or tissue.

In this paper we discuss a methodology to extract quantitative information about the distribution, density, size and locality of the photosensitizer accumulated in tumor cells or tissue from a stack of microscopical confocal laser scanning images.

2. CLINICAL SETUP

Although first treatments in PDT have been performed at the Department of Gynaecology and Obstetrics of the University Hospital in Zurich, this modality is at an early stage. This clinic has established a versatile system for both research and clinical applications in the field of PDT [2, 3, 4]. This setup allows to study biomedical processes during photodynamic activity for a broad range of conditions, extending from the patient down to subcellular structures. Investigations at the microscopic level will be performed in true three dimensions with living cells, colonies and tissue specimens in vitro. This is possible by a combination of confocal laser scanning microscopy (CLSM) which allows the non-destructive optical slicing of a probe, and specific hard- and software which has been developed at IBTZ at the University and ETH in Zurich.

3. CONFOCAL LASER SCANNING MICROSCOPY

In a CLSM a finely-focused laser spot is arranged in such a way that it coincides with the back-projected image of a point detector forming a confocal arrangement [6, 7]. The specimen is scanned through this confocal spot in three dimensions in order to generate a stack of image. The main benefits from this technique are three fold:

- an improvement in resolution by a factor of up to two over conventional light microscopy.
- a decrease in scattered light strength which results in an additional resolution improvement.
- optical sectioning capability by rejecting out of focus information thus allowing systematic investigations of thick specimens.

The CLSM setup used consists of a Leica True Confocal Scanner (TCS 4D). The resulting stack of images contains up to 140 planar slices with a minimal lateral resolution of ~170nm at 512 by 512 pixels and a corresponding slice thickness of about ~600nm[1]. The signal strength is digitized into 256 grey levels.

4. IMAGE PROCESSING SOFTWARE: QUASIA3D

The CLSM technique offers a precise depth discrimination combined with an enhanced lateral resolution not attainable with conventional light microscopy. To be able to

1 for an emission wavelength of 514nm. The lateral as well as the on-axis resolutions in a CLSM are dependant of the emission wavelength of the fluorescence dye and the variable pinhole diameter of the CLSM..

analyze and visualize the resulting stacks of slice images the concept of a dedicated volume processing software with a strong emphasis on the demands of CLSM has been defined.

QUASIA3D (QUantitative Analytical System for Image Acquisition in 3D) addresses in particular two problems which are inherent to volumes generated by CLSM:

- a high number of detected objects per volume which when visualized give rise to a large number of polygons (~$5*10^5$ and more) each with a small number of pixels (<30).
- numerous parameters influence the imaging process in CLSM and lead to distortions of the volume information. The most important problems are:
  - complex light absorption processes for excitation and emission
  - local sensitivity due to position dependant relative aperture
  - possible bleaching effects of photosensitizers
  - light scattering
  - low SNR due to the extremely fine focus volume

5. QUANTIFICATION SCHEME IN 3D

The main specification of QUASIA-3D for the quantitative approach is to deduce fluorophore content from the recorded brightness of a confocal image stack. The quantification process relays on the fluorometry equation:

$$j_{floresence} = j_{irradiation} \cdot \rho_{fluorophor} \cdot \eta_{fluorophore}$$

with

$j_{floresence}$: Emitted (observed) flux density

$j_{irradiation}$: Illumination spatial light flux density total

with

$\rho_{fluorophor}$: Photosensitizer dye concentration

$\eta_{fluorophore}$: Photoefficiency of the dye for the specific irradiation wavelength

If we consider for a moment a fibre fluorometer which records the total flux from an object in space we find:

$$flux_{total} = \frac{\Omega}{4\pi} \eta j_{irradiation} \eta_{fluorophore} \int_{Volume} \rho_{fluorophore} dx^3$$

$$= \frac{\Omega}{4\pi} \eta_f \cdot j_{irradiation} \cdot \eta_{fluorophore} \cdot m_{fluorophore}$$

with Ω the space angle covered by the fluorometer, η_f the gathering efficiency of the fibre tip and m the mass of all the fluorophores. The situation for a microscope is similar. With the numerical aperture NA = tan(α)

$$\Omega = \int_0^{\alpha} 2\pi \sin(x)\, dx = 2\pi\left(1-\cos(\alpha)\right)$$

$$= 2\pi\left(1-\sqrt{\frac{1}{1+NA^2}}\right)$$

the total recording efficiency is

$$\eta_f = \sigma_{Sensor} 4 \frac{n_{oil} n_{lens}}{\left(n_{oil}+n_{lens}\right)^2} 4 \frac{n_{oil} n_{cover}}{\left(n_{oil}+n_{cover}\right)^2} 4 \frac{n_{solvent} n_{cover}}{\left(n_{solvent}+n_{cover}\right)^2}$$

with σ the sensitivity of the microscope's registration sensor, n_{xx} the refraction indices of the various media (immersion oil, lens, cover glass).

Based on a known σ_{Sensor} the reconstruction for an ideal microscope would be trivial. Due to several distortions the CLSM observations are far from ideal for quantification purposes [8]. The list shows various influences ordered in

decreasing importance:

a) *Diffraction loss* . The media and the specimen exhibit inhomogeneous refraction indices, this introduces an elongation of the focal spot (spherical aberration). Combined with the illumination and registration process, this leads to a massive light loss in thick specimen, rendering sound observation almost impossible.

b) *Fluorophore decay (photobleaching).* Each fluorophore has a specific mean-excitation lifetime, i.e. it shows a probability p to be destroyed by an excitation process.

c) *Aperture function of the microscope.* A scanned voxel does not represent a cubic area of space over which the light flux has been integrated (scanned). It is a complicated and geometrically extended structure from which the light has been gathered. Mathematically this 'resolution function' is referred to as the *point spread function* (PSF) $\delta(x,y,z)$. It describes how the instrument accumulates its samples. The response I' for an original intensity I at a specific coordinate (x,y,z) is

$$I'(x_0, y_0, z_0) = \int_{R^3} I(x_0+x, y_0+y, z_0+z)\, \delta(x,y,z)\, dx\,dy\,dz$$

d) *Light scattering in the specimen.* Solid particles bend the light flux both for irradiation and emission. This has exactly the same effects as the spherical aberration discussed in a).

e) *Attenuation.* Specimens are not perfectly transparent, they consume a certain amount of light due to cross-excitation and thermal oscillation. The resulting effect is the same as a) and d).

f) *Excitation loss.* Irradiated light is absorbed by fluorophores during excitation. This means that areas behind bright zones appear darker. The influence is related to a), d) and e).

The orders of magnitude in falsification of the final results vary considerably between the different effects. With 20 µm thick specimen slices a) may cause for the deepest layer a loss of up to 75% of the total light. In a standard setup b) is less destructive. For a large number of layers it may consume an unlimited fraction of the efficiency (e.g. more than 80% for a stack of 100 slices). c) is harmful especially for fine structures, but as a rule of thumb it is proportional to the oversampling. d) and e) are difficult to separate from a) and b), but are reported to be less than 10% for 20µm and about $2 \cdot 10^{-3} \cdot$ zoom factor of objective, (20% for a 100x objective). Finally f) is less than 5% even for extended and bright volumes under observation.

Note that a) and d) have no meaning at all for non-confocal arrangements, b) almost none, c) is much simpler and of inferior magnitude, whereas e) and f) remain unchanged. This explains the much better quantification behavior of 2D fluorescence microscopy.

All these effects which degrade the quantification process can be accounted for, if they are precisely determined. A general reconstruction scheme may be pseudo-coded as follows:

Read specimen data
(attenuation, scatter, refraction mean & variance)
Read fluorophore data
(photoefficiency, bleach, wavelength)
Read instrument data
(sensitivity, NA, PSF)
ResetCounters
(scatter, attenuation, bleach, excitation)
FOR each layer DO
GetLocalData

(scatter, attenuation, bleach, excitation);
UpDateCounters
(scatter, attenuation, bleach, excitation);
AmplifyImageDataBy
(scatter, attenuation, bleach, excitation);
Adjust
(PSF)
END

This reconstruction algorithm fails due to the unavailability of the required data and due to the enormous computational effort which is needed for each *UpDateCounters* operation. Each update step comsumes computations proportional to the number of voxels in the image data set.

Consequently QUASIA3D solves the reconstruction problem with two different approaches. The first one implements efficient models and simplifications for the various effects in order to complete the restoration process in an acceptable amount of time.

The second task is to measure the missing parameters needed for the restoration. QUASIA3D implements a 3 stage model to gather these data sets:

1) The parameters of the confocal microscope such as wavelength sensitivity, focal spot geometry, PSF and objective features like distortion and local brightness response are collected via the sampling of phantom specimens.

2) Dye properties are measured with the help of pure solutions. Excitation attenuation and bleaching sensitivity are estimated. To gauge the bleaching sensitivity as a function of temperature and pH (environmental influence), the specimens have to be prepared accordingly. In a standard setup, the temperature is kept constant and therefore no temperature dependency has to be known.

3) Specimen properties are very difficult to separate in a microscope. If no external devices can be applied to distinguish between scattering and attenuation, experiments with fluorescent microspheres of precisely known shape have to be performed. The scattering effect shows frequency proportional strength. Thus scanning with different wavelength allows to differentiate between spherical aberration and the scattering effects. Attenuation versus scattering and aberration can be discriminated through the use of different aperture sizes. With high apertures attenuation becomes a more dominant effect. For a standard setup, no discrimination at all is required. The mixture of aberration, scattering and attenuation may be unified in an *absorption term*, which is typical for one excitation wavelength and one tissue type.

QUASIA3D uses this approach to build up its *specimen database*. Various image segmentation algorithms support the user in identifying the different tissues and gather absorption data for these in a semi-automatic way.

Obviously QUASIA3D is neither interactive nor thoroughly automated. The calibration procedures are semi-interactive in the sense that the operator must identify a set of representative specimen samples for the 3rd stage, but the reconstruction process runs without interaction solely based on the acquired data. This is very desirable, since the execution time for a reconstruction of an extended image stack takes up to 15 minutes - despite of all speed-up algorithms.

6. RESULTS

Worst case mathematical simulations which take all discussed effects (including cover glasses related) into account predict a 34% error bound for the acctual dye concentration

forecast.

In an experimental setup known tissues and precision reference beads were used to build up the tissue-specific correction database. QUASIA3D's algorithms were then used to estimate the dye concentration of phantom specimens consisting of tissue of the same type submerged in photosensitizer solutions of varying concentration. The light loss between the first few slices and slices far under the phantoms varied between 50% to 80%. For all slice levels QUSAIA3D's dye concentration estimation was within a 15% error bound.

7. CONCLUSIONS

A critical aspect for the success of PDT as a viable cancer treatment modality is besides a thorough understanding of the basic mechanisms involved in this technique, the ability to select and apply optimal tumor treatment conditions. This includes, as a prerequisite, the quantification of the local sensitizer dye concentration within cells or cell colonies.

Confocal laser scanning microscopy offers an efficient way to acquire 3D-image data sets of tumor cells or tissue for postliminary quantitative analysis. This paper outlines the various impeding effects which need to be accounted for if photosensitizer dye concentrations are estimated from stacks of CLSM data.

To this end an elaborate set of models and procedures were devised and implemented which allow to build an instrument-, dye- and tissue specific correction database. This database is then used to reconstruct the CLSM image stacks.

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