In vivo detection of circulating tumour cell clusters by photodiagnostic spectroscopy

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ABSTRACT

Background: We demonstrate a new diagnostic method, Photodiagnostic Infrared Spectroscoppy (PDIS), which is able to detect circulating tumor clusters and circulating tumor cells in the circulatory system.
Methods: The PDIS method is based on photodiagnostic physics and high resolution spectroscopy and is using calibrated spectroscopic data for the diagnostic analysis of the blood screening spectra. Using Confocal Laser Scanning Mikcroscopy the Indocyangreen-uptake of different cell lines of breast cancer cells is studied.
Results: The PDIS supplies calibrated diagnostic data about the presence or absence of CTCs and CTC clusters in the bloodstream with a sensitivity of 98 %. Therefore, the PDIS is suited to control the blood of cancer patients with respect of CTC and CTC clusters with an resolution of one CTC and one CTC cluster per blood volume. PDIS distinguishes the different phenotypes of CTC clusters.
Conclusion: Circulating tumor cell slusters play a key role in the metastatic process and are formed only in solid tumors, they are appropriate objects to validate cancer treatments and to recognice cancer formation. The PDIS is a calibrated diagnostic method which allows to evaluate the results of solid tumor treatments and of chemotherapy treatments and to optimize the individual cancer treatment strategies.

1. Background

Metastases are the main cause of cancer-related deaths; however, the mechanisms underlying metastatic spread are not completely understood. It is a complicated, multistep process that requires detachment of cancer cells from the primary tumour, intravasation into the bloodstream, the survival of tumour cells and tumour clusters in the bloodstream, and finally extravasation to distant organs [1]. Besides circulating tumour cells, tumour clusters seem to play a key role in the metastatic process [2]. In 1954, Watanabe showed that these clustered cells have high metastatic potential [3]. More recent studies indicated that clusters have distinct features compared to single tumour cells, including phenotype, gene expression signature, and dissemination mode. Thus, establishing the role and significance of circulating tumour cell clusters in the spread of cancer is of extreme importance. Circulating tumour cell clusters are defined as a group of more than two or three tumour cells with strong cell–cell contacts moving in the blood circulation and in the lymphatic system. They are rare, but highly metastatic. Despite representing only 2–5% of all circulating tumour cells, clusters were shown to form about 50 % of breast cancer metastases, and their metastatic potential was estimated to be 23–50 times higher than that of single cells [4]. Circulating tumour cell clusters represent a conglomerate of tumour cells and other types of cells including platelets, immune cells and cancer-associated fibroblasts. It seems that tumour clusters are epithelial-mesenchymal hybrids and therefore possess enormous plasticity [5]. This composition provides a local microenvironment that is thought to protect tumour clusters from death in the circulation by minimising shear stress and immune attack, and facilitating colonisation [6]. Au et al. have shown that clusters can actually traverse capillary-sized vessels [7]. It appears that upon constriction, tumour cell clusters undergo rapid reorganisation, forming a chain-like structure that reduces hydrodynamic resistance and allows them to pass through a small vessel. As concequence, the tumor cluster chains move preferentially parallel oriented to the vessel axis to maintain the lowest hydrodynamic resistance. This reorganisation was shown to be reversible, since after exiting constriction, clusters rearrange into their typical organisations. Aceto et al. [4] have demonstrated that the invasive potential of tumour clusters is higher than that of single tumour cells. Cheung et al. obtained similar results and estimated that approximately 97 % of metastases arise from clusters [8]. Other data suggested that intravascular aggregation and proliferation could be excluded as potential sources of tumour clusters due to the unfavourable conditions present in the bloodstream [9]. Very recently, Gkountela et al. [10] demonstrated that - in strong contrast to circulating tumour cells - the pattern of transcription factors in tumour clusters reveals similarities to embryonic stem cells along with

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increased proliferation. However, some cluster processes are still unknown regarding their genesis,transit and settlement and about the precise cellular and molecular mechanism. Nevertheless, the clinical data so far indicate the prognostic value of CTC cluster analysis in predicting treatment resistance and survival outcomes in cancer patients. [16]

Tumour clusters are of particular diagnostic importance because they only occur when a primary tumour exists. Tumour clusters thus may serve as indicators for the formation and existence of primary tumours, and conversely, they may also serve as indicators for complete or incomplete removal of primary tumours [4], as long metastases have not been formed. Cancer metastases arise mainly from circulating tumour cell clusters. Therefore, they should be the most important targets for metastasis prevention and reduction [8].The existing liquid biopsy methods of circulating tumour cell detection, like Cell Search [12], cannot be applied as reference for tumour cluster enumeration because they do not preserve their morphological and molecular status and are sensitive only for epithelial cells. Clearance rates of the labeled clusters and single CTCs from the bloodstream were established in mouse experiments by *in vivo* flow cytometry [7]. A photoacoustic *in vivo* method for circulating melanoma tumor cell cluster detection is described in [15].

There is no gold-standard method of detecting tumour clusters that enables a comparison of results obtained by different methods. Therefore, there is an urgent need for a standardised method for isolating and detecting circulating tumour clusters [2]. Photodiagnostic Infrared Spectroscopy (PDIS) is based on photodynamic physics, which was discovered and described for the first time by Jablonski in 1933 [11]. Photodynamic excitation is a physical principle that is explained in the Jablonski Diagram. The most important characteristic feature of a photodynamic process in general, and with liposomal indocyanine green (ICG) as a photosensitiser in particular, is the simultaneous emission of fluorescence and phosphorescence radiation. This is a kind of "fingerprint" and proves without a doubt the photodynamic origin of the signals. Therefore, in spectroscopic peak analyses, photon peaks generated by the environment can be clearly distinguished from photon peaks generated by a photodynamic process. The internal conversion of photon energy to heat can be neglected, it does not influence the detected signature of the signals. The amount of heat is very low due to the low excitation energies of about 10 mW/cm2 and the low concentration of ICG. We have used ICG as a specific liposomal formulation, which has an absorption wavelength of 785 nm, a fluorescence wavelength of 830 nm, and a phosphorescence wavelength of 940 nm, as reported by Bäumler et al. [13]. The advantage of liposomal ICG compared to normal ICG, which is used in angiography, is the much higher lifetime of liposomal ICG in blood and tissue ($\sim$ 36 h) compared to normal ICG ($\sim$ 10 min). After intravenous injection, normal ICG binds within $1-2$ sec almost completely (98 %) to serum proteins, therefore an accumulation in peripheral tissue or organs or even tumors is practically impossible.

2. Materials and methods

Methods for *in vivo* detection of circulating tumour clusters do not exist. Due to the lack of evidence-based reference procedures and standards regarding *in vivo* tumour cluster detection, we have calibrated the PDIS.

Fig. 1 shows the scheme of the flow calibration apparatus.

An optical fibre detector is placed within a flexible silicone tube 4 mm in diameter and 40 cm long, representing a mid-sized vein. The optical fibre detector is coupled to the high resolution-spectrometer. The whole assembly is placed in a clean-room box, maintained for measurements at a constant temperature of 37 °C and kept under dark conditions during measurement. The upper reservoir, with a volume of 500 mL cell nutrition solution, contains a port for cancer cell injection and a port for the optical fibre lead through. At the bottom of the upper
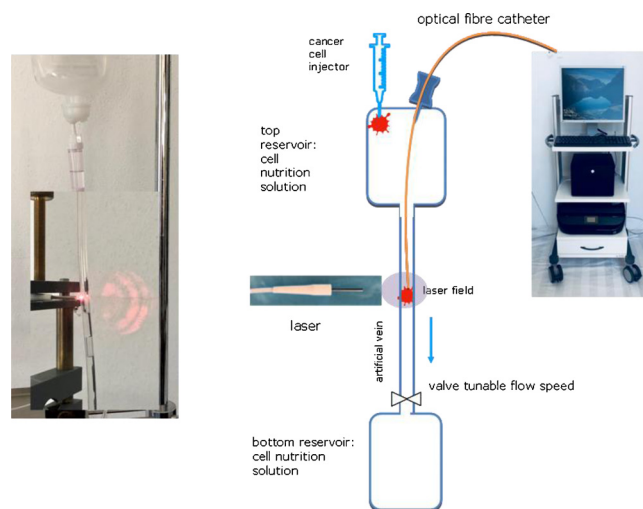

Fig. 1. Flow calibration apparatus for circulating tumour cells and clusters.

reservoir, a flexible silicon tube is arranged that connects the upper and the lower cell nutrition reservoirs. The cell nutrition solution flows in the flexible silicon tube from the upper to the lower reservoir. A valve on the lower reservoir is used to set the flow speed; in all experiments, the flow speed was kept constant at 10 cm/s. At the end of the optical fibre detector, an external laser source, emitting at 785 nm, is placed to generate an infrared light field within the silicon tube. When an ICG-incubated tumour cell or tumour cluster flows through the light field, fluorescence radiation at 830 nm and phosphorescence radiation are emitted and guided via optical fibre to the spectrometer. After 4 h incubation in 15 µM ICG solution, the cells, which were plated in a 6-well tissue culture plate at a concentration of 10 cells/well, were injected into the upper nutrition reservoir. The ICG uptake of MDA-231 cancer cell clusters was studied by haematological investigation using Confocal Scanning Laser Microscopy (CSLM). CLSM images were acquired by a VisiScope (Visitron Systems, Germany). Liposomal ICG was purchased from Burg-Apotheke, Koenigstein, Germany; the spectroscopic device was a C9505CB with a detection range from 400 nm to 1100 nm, produced by Hamamatsu Ltd, Hamamatsu, Japan. The laser catheter was purchased from Webermedical GmbH (Lauenfoerde,Germany); the numerical aperture was 0.3. The recorded spectra were statistically analysed with respect to peak emission wavelength (nm), peak "Full Width at Half Maximum" (FWHM)-broadness (s), peak intensity (counts), and peak origin (simultaneous fluorescence and phosphorescence emission).

3. Results

3.1. ICG uptake

Fig. 2 illustrates the uptake of liposomal ICG after 4 h in 15 µM solution concentration. The liposomal ICG accumulated inside the MDA-231 breast cancer cell, marked by the red colour. The cell nuclei were not affected.

The infusion concentration of liposomal ICGwas always 15 µM. In contrast, human leukocytes exposed to 15 µM ICG solution did not show any photosensitiser accumulation [14]. Fig. 2 shows clearly that liposomal ICG was accumulated in breast tumour cells and clusters at diagnostic concentrations of 15 µM. Hence, the prerequisite for photodiagnostic excitation of the breast cancer cells, as well for diagnostics for therapy, is fulfilled in light of these results. Our haematologic investigations with normal human leukocyte cells have shown that these cells do not accumulate liposomal ICG at concentrations of 15 µM. [14] Therefore, there are two components in the bloodstream which emit 830 nm fluorescence: on the one hand, there is the infused liposomal
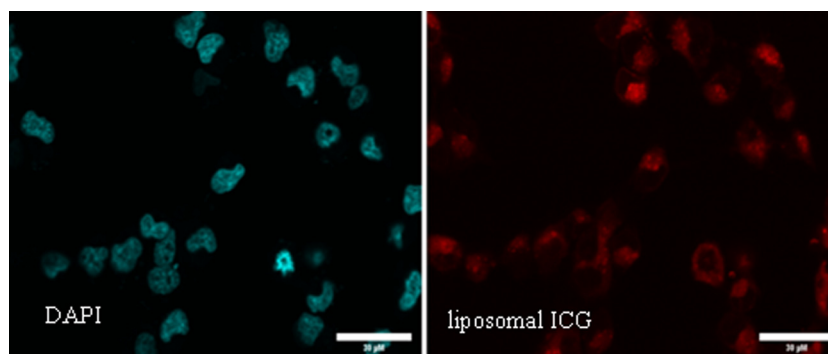
Fig. 2. CLSM image showing the ICG uptake of MDA-231 breast cancer cells incubated for 4 h at a liposomal ICG concentration of15 μM. The cells were counterstained with DAPI stain.

ICG, which is to 98 % bound to plasma proteins such as albumin and which forms a constant, continuous fluorescence radiation background. On the other hand, there are the low numbers of circulating breast tumour cells and tumour clusters, which have accumulated the liposomal ICG and which supply discrete fluorescence signals upon excitation. This is how it is possible to unambiguously distinguish the continuous background from the discrete tumour cluster peaks in PDIS blood screening. Before starting the PDIS blood screening, a" reference" light intensity is measured within 10^{-3} s. This reference spectrum is permanently subtracted from each "verum" spectrum (every 10^{-3} s) measured during the screening cycle of 10^3 s duration. As a result, only the additional fluorescence and phosphorescence peaks from tumour cells and clusters are recorded above the noise.

3.2. Calibration spectra

Fig. 3 shows the calibrated fluorescence peaks of a single MDA-231 breast cancer cell (A), a single tumour cluster cloud consisting of seven MDA-231 cells (B), and a breast cancer cluster chain consisting of three MDA-231 cells (C)

The calibration procedures were performed in a dark environment. The CTC clusters were externally prepared by adding plakoglobin to the tumor cell suspension. After passing the laser spot, the single clusters werer filtered out by a microcavity array filter and investigated by CSLM, to correlate the cluster morphology and the corresponding cluster fluorescence. When passing the 785 nm laser, the ICG-incubated cancer cells emitted 830 nm fluorescence peaks, which were guided to the spectrometer via optical laser catheter. The DMEM nutrition solutions used here were optically inert. The spectroscopic background radiation was compensated for with a special subtracting software, as described above, so that the starting intensity was always near the zero-fluorescence intensity value. The advantage is that only the photons emitted from the moving tumour clusters are displayed above the spectral noise. The flow speed was held constant at 10 cm/s, which is close to the blood flow velocity in the peripheral vena basilica, which has always been used in clinical PDIS screenings. Fig. 3(A) shows that as long the moving breast cancer cell is outside the light field, only small intensity fluctuations (statistical noise) near the zero-intensity line are recorded. If the cancer cell is directly in front of the optical fibre window, the intensity of the fluorescence steeply increases from zero to a maximum of about 2300 counts. When the cancer cell is leaving the light field, the intensity I f(d) decreases exponentially, according to the formula $1/d^2$ (where d = distance).The FWHM of the peak is of about 0.5 s +/- 0.2 s, in accordance with the flow speed of 10 cm/s. From a moving object, we have to expect an exponential decay of the fluorescence intensity according to the distance law if the object is leaving the light field. This is exactly what the peak in Fig. 3(A) confirms. As result, we get a calibrated peak-parameter set for a single moving MDA-231 cell, which represents, as a reference, the optical signature of a single breast cancer cell:

    emission wavelength: λ_F = 830 nm, λ_P = 940 nm
    peak broadness: FWHM = 0.5 +/− 0.2 s
    peak intensity: ~ 2500 counts +/− 200 counts
    peak shape: exponential intensity decay
    peak origin: t (Peak$_F$) = t (Peak$_P$)= photodiagnostic origin

It must be pointed out that in all 120 calibration experiments, almost constant peak intemsities for all MDA 231 cells were measured Obviously, the liposomal ICG uptake of breast cancer cells is relatively stable and not significantly different in blood and in calibration experiments. We never found peak intensities of single breast cancer cells above the limits, given in the peak parameter set.

The PDIS calibration peak of a moving tumour cluster cloud of seven MDA-231 cells in Fig. 3 (B) shows a peak, whose parameters are:

    emission wavelength: λ_F = 830 nm, λ_P = 940 nm
    peak broadness: FWHM = 0.5 +/- 0.2 s
    peak intensity: ~ 20,000 counts +/- 200 counts
    peak shape: exponential intensity decay
    peak origin: t (Peak$_F$) = t (Peak$_P$)= photodiagnostic origin

The only difference in the parameter set of the peak in Fig. 3 (A) is the peak intensity, which is 7–8 times the peak intensity of the single cell. In separate experiments, we have determined that the fluorescence intensity depends on the amount of ICG, which shows a linear behaviour. Therefore, we can estimate the number of cells contained in a tumour cluster cloud.

The PDIS calibration peak of a moving tumour cluster chain of three MDA-231 cells in Fig. 3 (C) shows three sharp consecutive peaks that indicate that the cluster had a chain-like orientation parallel to the optical fibre while passing the optical fibre window. This explains the peak distribution of three close consecutive peaks. The fluorescence peak represents a superposition of three bound circulating tumour cells with exponential intensity decay and a peak FWHM of about 1.6 s, which is three times the FWHM of a single cell. If the cluster chain had an orientation perpendicular to the optical fibre, the peak signal would appear as tumour cluster cloud, which is of similar clinical importance. The parameter set of the cluster chain passing parallel to the optical fibre is:

    emission wavelength: λ_F = 830 nm, λ_P = 940 nm
    peak broadness: FWHM = 1.6 +/− 0.2 s
    peak intensity: ~ 2000 counts +/− 200 counts
    peak shape: exponential intensity decay
    peak origin: t (Peak$_F$) = t (Peak$_P$) = photodiagnostic origin

The results of the calibration experiments are summarised in Table 1

The two phenotypes of tumour clusters can be distinguished by their peak-broadness (FWHM) and their peak intensities compared to the calibrated optical reference signature of a single tumour cell. It should be pointed out that the phenotype of the tumour clusters is not stable; it can reverse interchangeably from cloud to chain and vice versa [7]. Even so, the clinical relevance is not dependent upon cluster
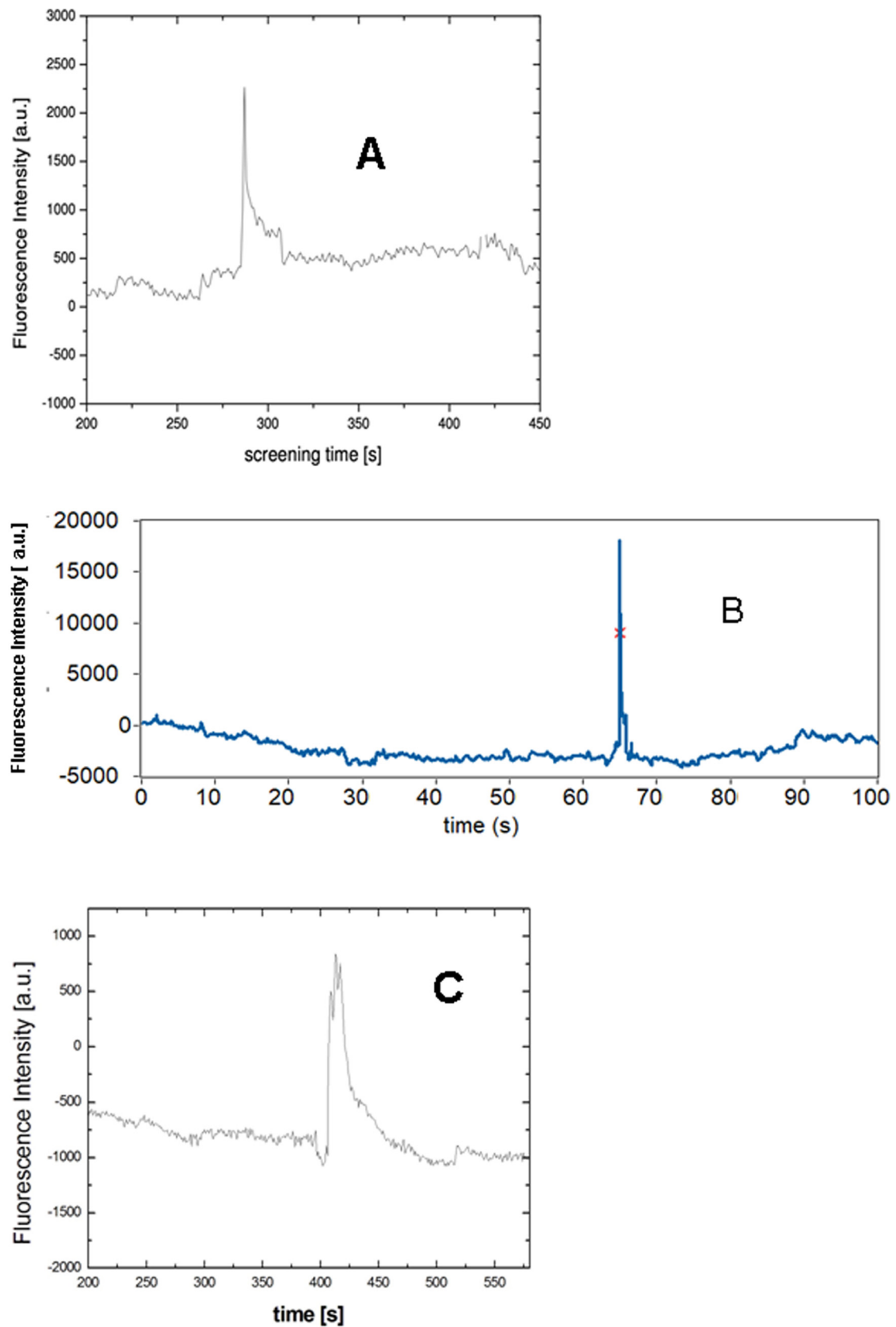
Fig. 3. Calibrated fluorescence peaks of a single MDA-231 breast cancer cell (A), a single tumour cluster cloud consisting of 7 MDA 231 cells (B) and a breast cancer cluster chain, consisting of three MDA 231 cells (C).

Table 1
Statistical analyses of the breast cancer cell calibration.

	average of 120 MDA 231 tumor cells	average of 120 MDA-231 tumor cluster clouds	average of 120 MDA-231 tumor cluster chains	Statistical significance
Emission wavelength/nm	830 nm ± 4 nm	830 nm ± 4 nm	830 nm ± 4 nm	$p < 0.001$
Peak FWHM /s	0,55 s ± 0.2	0,74 s ± 0.2	1,63 s ± 0,2	$p < 0.01$
Peak intensity/counts	2480 ± 200	20000 ± 200	2100 ± 200	$p < 0.01$
Peak decay	exponential	exponential	exponential	$p < 0.001$
Peak origin	photodiagnostic t(Peak$_F$) = t (Peak$_P$)	photodiagnostic t(Peak$_F$) = t (Peak$_P$)	photodiagnostic t(Peak$_F$) = t (Peak$_P$)	$p < 0.001$

morphology. When a tumour cluster, chain, or cloud is detected in the bloodstream, there must be a solid tumour in the body, and there is a significant risk of formation of metastases.

3.3. Circulating tumour cluster detection

After infusion of 15 mg liposomal ICG in 100 mL 0.9 % NaCl, the optical laser catheter was placed into an arm vein (vena basilica) and the PDIS screening was started by activating the 20 mW, 785 nm extravascular laser, which was positioned above the optical fibre window. The PDIS spectrum was measured over 1000s with the assumption that during this time, the entire blood volume has passed through the optical fibre detection area. The PDIS blood screening of a patient with a stage 2 non-metastatic carcinoma of the left breast is shown in Fig. 4.

All peaks in Fig. 5 are of photodynamic origin; the corresponding phosphorescence peak for each occurs at exactly the same time. This largest peak also shows clearly exponential intensity decay. We can conclude that the blood screening spectrum of the breast carcinoma patient of Fig. 4 shows definitively the existence of circulating tumour cells and circulating tumour clusters in the bloodstream. To destroy these clusters, Photodynamic Therapy was performed using liposomal ICG. The photo-oxidative killing of tumour cells by ICG was reported by Bäumler et.al. [13]. After LED irradiation of a vein with an external 785 nm infrared LED radiation head of 40 mW power and 30 J/cm^2, the
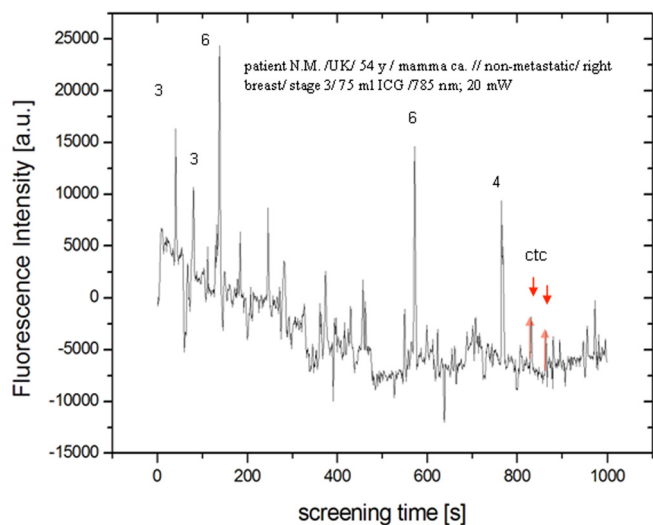

Fig. 4. PDIS blood screening spectrum of a patient with a stage 2 non-metastatic carcinoma of the left breast, measured 4 h after ICG infusion. The peak analysis was performed by determining the peak intensity and peak area of each peak in the figure comparison with the calibrated optical signature of a single tumour cell. The red downward oriented arrows show peaks that are in agreement with the single cell parameters, indicating circulating tumour cells. The other peaks indicate tumour clusters, where the numbers represent the number of cells contained in the cluster. The clinical importance of PDIS screening is that it can be shown without a doubt whether there are tumour clusters in the bloodstream of the patient, which must be destroyed immediately by PDT or chemotherapy before forming metastases.
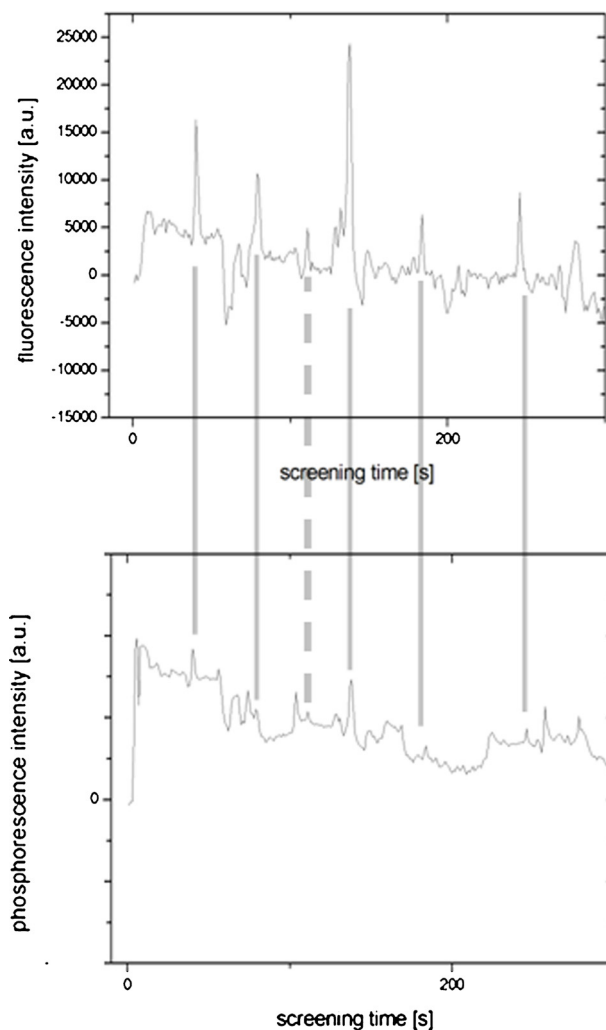

Fig. 5. Cutout of the fluorescence and corresponding phosphorescence blood screening of the non-metastatic breast carcinoma patient from Fig. 4. The cutout spectra between 0 and 300 s illustrate the simultaneous occurrence of fluorescence and phosphorescence peaks, which prove the photodynamic origin of the peaks. The dotted line represents a circulating tumour cell, while the other lines correlate with peaks of tumour clusters. The excitation wavelength was 785 nm; the laser power was about 20 mW. The optical fibre detector was placed in the vena basilica.

circulating tumour cells and tumour clusters were photo-oxidatively killed, and the final PDIS diagnosis of Fig. 6 showed only noise, with no peak-signals from circulating tumour cells or tumour clusters.

The decrease of the fluorescence intensity during the measuring cycle is mainly due to the photodynamic destruction of tumour cells and clusters, which decreases the ICG concentration in the blood. The influence of bleaching effects was determined in separate experiments that showed intensity reductions of liposomal ICG during the screening cycle due to bleaching effects on the order of 3%.
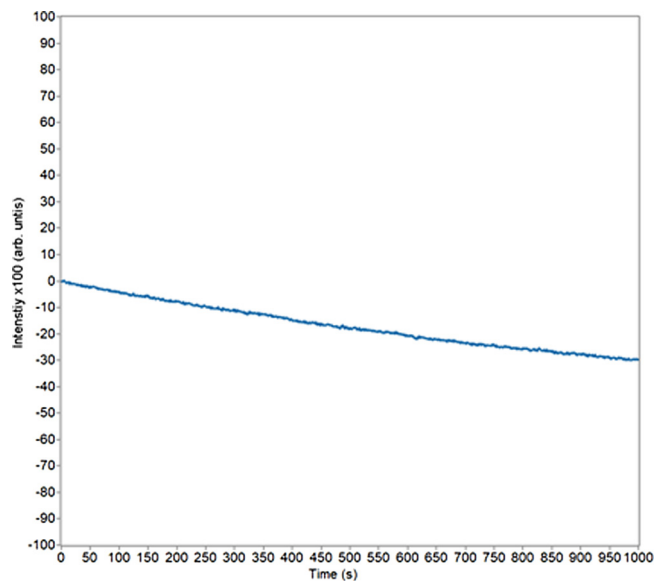
Fig. 6. PDIS spectrum of the breast cancer patient from Fig. 4 after photo-oxidative killing of the circulating tumour cells and tumour clusters by Photodynamic Therapy using liposomal ICG as photosensitiser. The spectrum does not contain peaks from circulating tumour cells or tumour clusters.

4. Discussion

The discontinuous emission of CTC clusters reported by Aceto et al [4] is in agreement with the PDIS screening shown in Fig. 4. It is obvious that the primary tumour emits the breast cancer clusters discontinuously.

Statistical analysis of 185 patients with non-metastasised breast cancer diagnosis after ICG infusion resulted in about 98 % sensitivity of circulating tumour cluster detection. In this case, "positive" wrong or" negative" wrong results can be practically precluded. It should be pointed out that PDIS diagnosis does not distinguish between the circulating tumour clusters of different primary tumors and therefore cannot be used to identify the type of the primary tumor. But it can be used to detect the presence of a primary tumor in the organism. Independend on the type of the solid tumour, the primary diagnostic information of PDIS is are there CTC clusters in the bloodstream or not?

The PDIS screening data contain precise, calibrated diagnostic information about the presence or the absence of the hazardous tumour clusters in the bloodstream. As consequence, PDIS screening allows to determine the clinical efficacy of any tumour surgery, radiation therapy, chemotherapy, photodynamic therapy and other cancer treatments. When metastasis already are formed, they are also emitting tumor cell clusters, which can be detected by PDIS. In this case, the PDIS controlled blood cleaning may help to reduce the metastasis spread in the organism.

Although demonstrated here only in breast cancer, PDIS diagnostics can be performed for all solid tumour types, provided that flow-calibrated data are available. PDIS data allow clinicians to confirm or to change the applied individual therapeutic strategy and to recognise the development of resistance against chemotherapeutics.

For healthy individuals without tumours, PDIS detects either zero or a very low number of circulating tumour cell peaks, similar to Cell Search [12]. However, we have never detected tumour clusters in the blood of healthy probands. The investigations of Aceto et al. [4] and Cheung et al. [8] have demonstrated that circulating tumour clusters are always formed in tumours and cannot be formed in vessels by aggregation. Therefore, tumour clusters seem to be appropriate indicators or precursors of the existence of a primary, solid tumour, and conversely, are appropriate indicators of the complete or incomplete destruction of primary tumours. When tumour clusters appear in the

bloodstream after breast cancer treatments like surgery or chemotherapy, the primary tumour has not been completely removed. PDIS allows physicians to control the dissolution of circulating tumour clusters and to maintain careful control over the presence of tumour clusters in the blood. The use of PDIS blood screening after finishing chemotherapy makes it possible to provide patients with calibration-based information about their status, thereby reducing psychological stress, fear, and uncertainty, which are all consequences of the current "wait and hope" principle.

Liposomal ICG is safe and free of side effects. We never observed any side effects within the 36 h time of permanency in the blood. The infused liposomal ICG concentration was of about 0.3 mg per 1 kg body mass. The procedure is easy to perform, since the infusion of the liposomal ICG is the only treatment step and is minimally invasive. Further flow calibrations of PDIS spectra for different cancer types and different approved photosensitisers like Photofrin are necessary and will be performed in the near future. Finally, it should be mentioned that -beyond cancer diseases- the PDIS can be applied for detection and destructionen of viruses in the blood. It was demonstrated recently by Keil et.al. [17] that the dye Riboflavin binds to corona viruses, which enables the detection and destruction by PDIS using 450 nm blue light emitters.

5. Conclusion

In summary, we have developed and calibrated the diagnostic PDIS blood screening method. PDIS supplies calibrated diagnostic data about the presence or absence of circulating tumour clusters, which, in combination with other diagnostic information, allows for fast and easy estimation of the success of applied cancer therapies. The PDIS procedure represents a new diagnostic method to monitor and control the most important transport channels for distant metastases formation with regard to circulating tumour cell clusters: the circulatory system and the lymphatic system. A PDIS blood screening can be performed and analyzed within 45 min. PDIS diagnostics allows for the verification of the results of surgery and chemotherapy, and for the targeting of treatment strategies for circulating tumour cluster dissolution to keep the blood of cancer patients free from tumour clusters and cells, and consequently, to prevent metastasis formation and to improve the overall survival. The PDIS method can be applied also for corona virus detection and destruction using Riboflavin as photosensitizer.

Declaration of Competing Interest

The author declares no conflicts of interest.

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