Detection of circulating tumor cells in samples from colorectal cancer patients using digital fluorescent microscopy and image cytometry

PhD thesis

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INTRODUCTION

Recently enhanced focus is set on the rare cells of the human organ (circulating tumor cells, physiologic and pathologic stem cells, fetal cells). Circulating tumor cells can be detected in peripheral blood samples by cytometry. The two main cytometry approaches are flow and image cytometry.

Slide based image cytometers are built around microscope optics with fluorescent light path and high resolution imaging capability. They provide similar data as flow cytometry (FCM) by digitally processing the cell images. For the detection of circulating tumor cells slide based image cytometry (SBC) has several advantages over FCM. It provides morphological and sub-cellular information. Cell location on the slide is recorded and based on the measured data selected cells can be relocated or examined with different imaging techniques. SBC can be utilized for relatively small specimens and its application for rare cell detection is proven. Samples can be re-stained and rescanned to acquire more parameters and the analysis of cell clusters and tissue is also possible.

The development of computer and CCD (charge coupled device) camera technology made possible about 20 years ago the appearance of slide based imaging cytometer systems with acceptable throughput and data handling capability. Since that time systems were developed based on Laser Scanning Cytometry (LSC), standard, enhanced wide-field and confocal fluorescent microscopes. LSC is the most widespread SBC solution.

For clinical use standard fluorescent microscopes are the most promising modalities. They are widely available; they have lower basic costs as other systems and they can be used for general microscopic work which lowers the costs of circulating tumor cell detection even further because no dedicated system is necessary.

In the recent years another imaging microscopy field developed very rapidly. Brightfield virtual microscopy or whole slide imaging (WSI) became commercially

available and starts to be established in pathology. Whole slide imaging means that complete sections, cytospins and smears can be digitized automatically in high resolution that is appropriate for diagnosis.

AIMS

The objectives were as follows:

1. The development of methods to use a fluorescent microscope for quantitative and stoichiometric cytometric measurements. This set of methods and software is called Scanning Fluorescent Microscopy (SFM).
2. To show on biological samples that SFM is capable to reliably detect rare cells. This is done by comparing the analytic accuracy of the developed SFM methods with FCM and LSC on high and low cell concentrations (between 1:1 to $1:10^7$ cell frequency) in artificial and clinical specimens.
3. The further development of the SFM methods to modify a fluorescent whole slide imager to be capable of quantitative and stoichiometric measurements to make the workflow effective enough for the clinical routine screening of colorectal cancer patients.

METHODS

Scanning Fluorescent Microscopy

Sample preparation

For testing and calibration of the system, 10 µm diameter cytometric calibration beads were used. For evaluation of clinical samples, residual samples from young, cancer free patients were used. Mononuclear cells were stained in Hoechst 33258.

For the correction of mercury arc lamp uniformity errors, a slide with evenly distributed FITC stain was prepared.

Scanning Fluorescent Microscope Hardware

The SFM includes hardware and software components. In this study a Carl Zeiss Axioplan 2 imaging MOT motorized microscope with Carl Zeiss Plan-Neofluar 20x/ NA 0.5 objective and AxioCam HRc camera was used.

SFM Software components

Autofocus module finds the best focus level for a field of view during scanning.

Slide viewer module provides the functionality of a virtual microscope. Displays the data recorded from the real slide.

Image processing module evaluates off-line the scanned digital slide. The first step of image processing is illumination none uniformity compensation. Every field of view is compensated according to the following equation. I' denotes the compensated image, I denotes the original, B the black reference and W the white reference image.

$$I'_{x,y} = (I_{x,y} - B_{x,y}) * \frac{W_{\max_{u,v}} - B_{u,v}}{W_{x,y} - B_{x,y}}$$

In the second step of image processing, images are thresholded. The following morphometric parameters are calculated for every cell: maximum diameter, minimum diameter, average diameter, area, and perimeter. The following fluorescence parameters are calculated for every cell: integrated fluorescence, minimum fluorescence, maximum fluorescence, average fluorescence, and fluorescence range.

Scatter plots, histograms and galleries can be used for the cytometric evaluation of the data derived from image processing.

Linearity measurement

For testing the system's linearity, a homogenous fluorescence bead sample was scanned multiple times with exposure times between 1,000 and 4,000 ms in 500 ms steps. The correlation between the integrated fluorescence's mean value and the exposure times was calculated.

Calibration beads with different intensities were scanned by SFM and measured on FCM. The ratios of fluorescence measured by both modalities were compared.

Comparison of SFM, FCM and LSC

High concentration samples for relative cell frequency determination

Peripheral blood mononuclear cells (PBMCs) were isolated from of young cancer free patients. HT29 and PBMCs cells were counted manually and mixed at different ratios (HT29 to PBMC ratios: 1:1, 1:2, 1:4, 1:8, 1:20, 1:50, 1:100, 1:500, 1:1,000) in three replicates. 100,000 – 200,000 cells were used per mixture. Anti human CD45 ECD and CAM 5.2-FITC antibodies and DNA specific fluorescent dyes TOTO-3 and Hoechst 33258 were used for staining. Samples were divided into two parts for FCM and SBC measurements. For SBC smears were placed onto a spot with a diameter of 5- 6 mm in the middle of conventional glass slide.

Low concentration samples for absolute cell frequency determinations

(SBC measurements)

CAM 5.2-FITC labeled HT29 cells were placed by micromanipulator on the slide smears contained Ficoll separated, CD45-ECD labeled, peripheral blood mononuclear cells.

Slides prepared from the blood of tumor bearing patients

Peripheral blood of colorectal cancer patients with different Dukes stages (B:2, C:3, D:5) were evaluated. Enrichment of HEA-125 expressing tumor cells was achieved

using MS cell separation columns (Miltenyi Biotech). After isolation in the magnetic field the enriched cell fractions were labeled for CD45 ECD, CAM 5.2 FITC, and nuclear DNA.

Flow cytometry

The analysis was performed on a FACScan flow cytometer with a 488 nm argon-ion laser for excitation. Fluorescence signals were detected at 530 nm (FITC) and 650 nm (ECD).

Laser scanning cytometry

LSC (Compucyte) was equipped with an Olympus UPLANFL 20x / NA 0.5 objective. Fluorochromes were excited by a 488 nm and a 633 nm laser and fluorescence signals were detected at 530 nm (FITC), 625 nm (ECD) and 670 nm (TOTO-3).

Scanning fluorescent microscopy

Fluorescent light was detected for Hoechst 33258 with Carl Zeiss filter set 02, for ECD staining with Carl Zeiss filter set 20 and for FITC Carl Zeiss filter set 10.

Visual Fluorescent microscopy analysis of rare cells

The manual screening and evaluation of low concentration specimens was performed by two independent observers using an Carl Zeiss AxioPlan 2 Imaging microscope equipped with triple path and single path filters for FITC, ECD and Hoechst 33258 staining and a Carl Zeiss AxioCam HRc camera.

Quantitative and stoichiometric fluorescent whole slide imaging

Samples

For testing and calibration of the system the same cytometric calibration beads were used as for SFM. For evaluation of clinical samples, residual samples from young, cancer-free patients were prepared in the same way as for SFM.

For correction of the illumination uniformity errors, a special compensation slide was prepared by the Fraunhofer Institute for Applied Optics and Precision Engineering IOF. On a glass slide in a 1.45 µm thick polymethyl methacrylate layer the following laser dyes were diluted: Coumarin 2, Coumarin 545, Rhodamine 6G, Rhodamine 101, Oxazin 4, Nile Blue, Rhodamine 800.

Hardware

A MIRAX MIDI automated digital microscope was used which can scan in brightfield and with extension described in this work in fluorescence. It had a slide loader mechanism for 12 slides, Zeiss 20x Plan-Apochromat, NA 0.8 objective. Zeiss filter blocks were used for DAPI (Filter Set 49), FITC (Filter Set 38HE) and Rhodamine (Filter set 43HE). For the quantitative fluorescent measurements a Carl Zeiss Colibri LED light source was used with 365 nm and 470 nm LED modules. For general scanning tests a Carl Zeiss HXP 120 metal-halid short arc lamp was used that can be fiber coupled to the Colibri lamp. For image capture a Zeiss AxioCam MRm Rev.3 monochrome camera was used.

Algorithms

Sample detection and localization.

MIRAX MIDI is equipped with a preview camera to grab a low resolution image from the slide to determine areas for imaging. The fluorescent imaging software requires to circle the sample with a continuous line on the slide with a black marker pen.

Sample mapping

Before imaging the sample is mapped. On grid points the sample is focused and exposure times are measured in every channel.

Focusing

To adapt to the varying light intensity the exposure time is continuously adjusted during focusing. Sharpness calculation is based on pixel value differences in an image. The

reliability of the focusing algorithm was tested by auto-focusing and manually checking 100 field of views.

Sharpness calculation

Sharpness value calculation is based on a pixel value differences histogram. After histogram generation its values are multiplied with the 5^{th} power of the histogram indices.

Image compensation

During acquisition of the digital slide every image is compensated using the following equation:

$$I'_{xy} = I_{xy} \frac{C_{max\,uv}}{C_{xy}}$$

I' denotes the compensated image, I denotes the original image and C denotes the compensation image. The x and y indices denote an image pixel, and u and v denote the coordinates of the brightest pixel of the compensation image.

To evaluate the effect of compensation 1200 of the brightest beads with 20 ms exposure time were scanned without compensation and their CV value was compared to the same bead population scanned with compensation for the system linearity measurements detailed later.

Image segmentation

The system uses thresholding for image segmentation. For a segmentation an upper and lower threshold can be defined and pixel values below and above those values will be excluded.

Quantitative and stoichiometric measurement

The following parameters are measured for each object: area, perimeter, shortest and longest diameter, shape factor, average pixel intensity and integrated fluorescence (IF).

System linearity measurement

The system's linearity was verified by three different methods. To measure exposure linearity beads were scanned with 5, 10, 15, 20 and 25 ms exposure time. To test single exposure linearity beads were scanned at once from a mixture of all the 5 intensities. To verify areal linearity beads and bead clusters were scanned and the area of different cluster sizes was correlated with the number of beads. The CV value of Hoechst stained lymphocytes was measured to assess usability on real samples.

Fluorescent virtual microscopy

For slide display the MIRAX Viewer software was used. The viewer has the following main functions: arbitrary magnification selection, panning, annotation handling, measuring and opening slides from a teleconsultation server on the Internet.

Measurement evaluation tools

Measurements can be evaluated by the scatter plot, histogram, gallery and data export tools of the HistoQuant package.

Results

Scanning Fluorescent Microscopy

Scanning

The usual time for movement, auto focusing and image capture for a field of view was 5±0.5 seconds; and 975 KB were required for the storage of an area of 430 µm * 320 µm. A 3.7 mm x 3.7 mm cytospin digitized in 3 fluorescent channels required 100 frames and without image compression 285 MB of storage area.

Compensation

Without shading compensation the CV value of the beads was 24.3% which dropped to 3.9% with compensation.

Linearity

The correlation between the mean values of the measured fluorescence and exposure time was 0.999963 ($p < 0.0001$).

The ratio of the two brightest bead population on FCM was 4.11 and on SFM 4.00. The ratio of the second and third population was 3.84 on FCM and 10.67 on SFM.

Compression

It was found that using standard JPEG, up to a compression ratio of 1:150, the CV does not change significantly. A general property of lossy image compression technologies, such as JPEG, is that the loss is in the resolution domain and not in intensity. Since the quality measurement is based on integrated fluorescence or intensity, the CV is good at high compression rates but the image quality is not acceptable. The best compromise in compression is between 1:50 and 1:100, in this range the CV and image quality is still very good but the size is reduced dramatically.

Focusing accuracy

The calculated depth of focus is 1.914 μm for a 20x N.A. 0.5 objective. The CV of single beads is 2.8 in the ideal focal plane and is the same in 2 μm range which is in correlation with the calculated depth of focus. As the focusing range is extended the CV is increasing. Up to 10 μm focal range the CV is below 4 and is acceptable for measurements.

Clinical sample

The CV of the Hoechst stained lymphocytes was 5.6.

Comparison of SFM, FCM and LSC

In this study the goal was to use for comparison the same sample for all three techniques. Therefore two different DNA dyes and ECD labeling was used with broad excitation (460-600nm) and emission (555-680 nm) spectrum to overcome the problem

of different excitation and emission spectra of the detection modalities. The FITC and ECD labeling was detectable by all three instruments. For nuclear counter staining TOTO-3 and Hoechst 33258 were detectable by the LSC (CV range 12-14%) and the SFM (CV range 6-8%) respectively. The relatively high CV value in TOTO-3 staining did not impede finding cells by the LSC. In our experiments we did not use permeabilisation and RNA-se treatment, so DNA staining could be heterogeneous and TOTO-3 could bind to RNA also giving broad variance in TOTO-3 fluorescence.

Relative cell frequency determination of high concentration samples by FCM and SBC measurements

In the measurements the most suitable fluorescence detection of cell surface markers were shown by the FCM followed by SFM and LSC. In the FCM measurements on the dot plots there are clearly distinguishable cell populations, which are less distinct with SFM and LSC.

Absolute cell frequency determination of low concentration samples by SBC measurements

The correlations between the number of placed tumor cells and that determined by the SFM and LSC were very high (r^2=0.96, $p<0.001$; r^2=0.95, $p<0.01$ respectively).

In the circulating tumor cell samples of seven patients the two systems showed similar results. In the other three cases LSC yielded a higher number of cells than SFM (749 vs 166, 355 vs 43, 61 vs 5). In these studies LSC overestimated the number of tumor cells.

Quantitative and stoichiometric fluorescent whole slide imaging

Focusing

For 93% of the field of views the automatic focus algorithm found the same focus level as manual focusing or difference was less than the depth of field. In 7 percent of the cases the difference was 0.8 μm.

Scanning results

The marker pen based sample detection worked reliably if the marking was at least 1 mm wide and fully connected in the area imaged by the preview camera.

Measurements: System stoichiometric linearity after compensation

The calculated correlation between exposure time and integrated fluorescence was 0.999491. The correlation between the manufacturer defined intensity ratios and the measured integrated fluorescence was 0.999548. The correlation between the average area of single, double and triple clustered bead populations and the number of beads in a cluster was 0.999998. The Hoechst stained control sample had a CV value of 6.4.

Conclusion

A motorized fluorescent microscope with the use of compensation slides and special image processing algorithms can be used for quantitative and stoichiometric cytometry measurements. Such a system provides comparable results with similar sensitivity and specificity to well established Flow cytometry and Laser Scanning Cytometry. Slide based image cytometry is superior to Flow Cytometry for rare cell detection.

With further development of the methods a fluorescent whole slide imager can be used for quantitative and stoichiometric cytometry measurements. The technical advancements of whole slide imaging over regular microscopy makes the clinical workflow more effective in terms of reliability, speed, manpower and costs. With these methods the clinical screening of colorectal cancer patients becomes possible.

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