Microarray analyzer based on wide field fluorescent microscopy with laser illumination and a device for speckle suppression

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Abstract: A microarray analyzer was developed to obtain images and measure the fluorescence intensity of microarrays at three wavelengths from 380 nm to 850 nm. The analyzer contains lasers to excite fluorescence, barrier filters, optics to project images on an image detector, and a device for suppressing laser speckles on the microarray support. The speckle suppression device contains a fibre-optic bundle and a rotating mirror positioned in a way to change the distance between the bundle butt and mirror surface during each mirror revolution. The analyzer provides for measurements with accuracy within ± 5%. Obtaining images at several exposure times allowed a significant expansion in the range of measured fluorescence intensities. The analyzer is useful for high throughput analysis of the same type of microarrays.

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1. Introduction

Biochips, also referred to as microarrays or biologic microchips, are used to analyze many parameters of a sample in parallel. Depending on the nature of the probes that are immobilized in the microarray elements, various tasks can be implemented, including the detection of specific nucleic acid sequences and their mutations, the study of certain gene expressions, immunologic analyses, infectious agent detection, etc [1–7]. Frequently, an analyzed sample is labeled with a fluorescent dye, and the fluorescence intensity of the various microarray elements after their incubation with the sample indicates the affinity of the sample to the molecular probes immobilized in the microarray elements.

The various methods developed for the excitation and measurement of microarray fluorescence depend on the technique used to manufacture a microarray and the type of analysis [8–12]. Typically, microarray analyzers are based on confocal microscopy, which is a scanning technique that utilizes a narrow laser beam to excite fluorescence of a small
microarray area, and the fluorescence intensity of the excited area is measured “point by point” using a precise scanning photomultiplier-based device [13–16]. Several companies manufacture scanning devices for microarray analysis, such as Agilent, Thorlab, Molecular Devices, Arrayit, etc., and their analyzer features are readily available in the review papers [see, for example 17].

Laser-scanning techniques for microarray analysis are popular because most microarrays contain probes, which are located in a monomolecular layer on a surface support that forms a two-dimensional (2D) structure with a low fluorescence intensity of individual elements. In a confocal-based analyzer, a field diaphragm placed on the image plane facilitates a high signal-to-noise ratio (SNR), which allows for the detection of single fluorescent molecules [18,19]. Meanwhile, the need for point-by-point scanning of the microarray lengthens data acquisition times and lowers equipment throughput. Moreover, the scanning technique requires precise mechanical parts, which lead to a higher price of the analyzer.

Another type of microarray analyzer, one based on wide-field digital microscopy, utilizes a defocused laser beam to excite the fluorescence of all objects located in the analyzer’s field of view (FOV), and an image of fluorescence microarray cells is obtained using an image detector (CCD or CMOS camera) [20,21]. The size of the analyzed microarray area is determined by the objective-ocular FOV and may vary from several square microns to hundreds of square millimeters. Analyzers based on wide-field fluorescence microscopy are sufficiently sensitive to quantitatively analyze the fluorescence intensity of microarray cells that contain fluorochromes. High-throughput analyzers based on this approach are essential for conducting serial and uniform tests. In Russia, this type of analyzer is recommended for testing large populations of patients infected with tuberculosis [22]. Moreover, because this type of analyzer can simultaneously obtain images of the objects located within its FOV, it may be used to monitor parallel kinetic reactions in various microarray cells [23].

Fluorescence excitation that uses a defocused laser beam is accompanied by speckles, which are areas with different levels of illumination brightness caused by the interference of coherent rays on a surface [24]. These speckles alter the illumination intensity of the microarray cells and therefore decrease the accuracy of fluorescence measurements of the microarrays that contain the fluorescent markers.

Multimode fiber-optic bundles with different individual fiber lengths can reduce speckle intensity. Using longer bundles with individual fiber lengths that vary more widely can improve speckle suppression [25]. Another method used for speckle suppression is based on liquid-crystal filters with a constantly changing crystal structure [26] or a combination of multimode fiber bundles with liquid-crystal filters or a microlens array [27–29]. Unfortunately, the speckle suppression coefficients in many of these devices do not provide sufficiently accurate quantitative measurements of microarray cell fluorescence intensities.

In our previous work, we used a combination of a fiber-optic-ring illuminator with a device that mechanically moved the fiber-optic butts, which were guided to an illuminating laser [30]. The ring illuminator increased the uniformity of illumination in the FOV, whereas the fiber-optic butts, which were guided to the laser diode, were subjected to reciprocating vibration. As a result, constantly changing speckle localization was observed at the butts of the fiber bundle coupled to the laser. An increase in fiber amplitude movement was accompanied by a reduction in the appearance of speckles. One drawback of the device is the need to mechanically move the fiber-optic ends, which reduces their life span.

Herein, we describe a fluorimetric microarray analyzer based on wide-field digital fluorescence microscopy, which allows for the collection of fluorescence microarray cell images at three wavelengths with 5% accuracy due to efficient speckle suppression.

2. Materials and reagents

The following laser diodes were used to excite microarray cell fluorescence: green (532 nm – 50 mW), red (655 nm – 80 mW) and infrared (IR) CNI MLL-III-750 (750 nm – 250 mW)
[31,32]. The excitation light was projected onto the analyzer’s FOV by mirrors rotated with RF-300FA-12350 motors (3500 rpm) [33]. The illumination intensity was measured using a power/energy meter, model 1825C [34]. Part of the laser power was lost while passing the optics between the light source and the sample, and the illumination brightness of the FOV measured by the power meter was approximately 0.3 milliwatt/mm² at 532 nm, 0.5 milliwatt/mm² at 655 nm, and 1.5 milliwatt/mm² at 750 nm.

The fluorescence was cut off from the excitation light using Semrock barrier filters 607 ± 35 nm, 716 ± 22 nm and 819 ± 22 nm [35]. The microarray images were projected onto a 12-bit CCD camera [36] with a SONY ICX285, 2/3-inch sensor using a pair of 50-mm f/1.4 Nikon objectives. Black and white photographic film Mikrat 300 [37] was used to test speckle suppression.

The other mechanical parts were manufactured in a workshop. The microarrays that contained the labeled ATGCATGC oligonucleotides were manufactured as previously described [38]. The labels (Cy3-NHS Ester PA13101, Cy5-NHS Ester PA15101 and Cy7-NHS Ester PA17101) were purchased from Amersham. The images were processed utilizing Imageware software [39].

3. Results

3.1 Design of the analyzer

The analyzer contained an illumination device and a device for obtaining a fluorescent microarray image. The illuminating device contained laser diodes, fiber-optic bundles and a rotating mirror to distribute the excitation light within the FOV. The device used to obtain images contained objectives, barrier filters and a CCD camera. A photograph of the analyzer without the front and sidewalls and upper lid is shown in Fig. 1(A); a schematic representation of the analyzer is shown in Fig. 1(B).

![Fig. 1. Photo of the analyzer without front and sidewalls and an upper lid (A), and schematic representation of the analyzer (B). The drawing shows three illuminating devices for three wavelengths, a microarray holder, an upper objective, a focusing device and a CCD camera.](image)

The microarray image was projected onto the image sensor of a CCD camera using a pair of Nikon photographic objectives positioned antiparallel to each other. Because two identical objectives are used in the analyzer, the total magnification of the object at the sensor is 1:1, and the size of the FOV is equal to that of a CCD sensor, which in our analyzer is 8.77 x 6.6 mm. The objectives have a constant focus in the visible spectral region; however, additional focusing in the IR region is required. Therefore, measurements in the IR region (switching the excitation lasers) were accompanied with additional focusing.
The optical scheme used in the analyzer does not allow for uniform illumination of microarray cells located in different parts of the FOV. This non-uniformity is caused by the appearance of speckles arising from the interference of coherent rays on a surface and by unequal illumination of the FOV with defocused laser beams. Therefore, special measures are required to take into account this non-uniformity of the FOV.

3.2 Speckle-suppression device

Illumination via defocused laser beams caused local differences in the excitation brightness within the FOV (speckles) and decreased the accuracy of the fluorescence measurements of the microarray cells. The size of the speckles depends on the light wavelength and may vary from several microns to several millimeters. Figure 2(A) shows a device utilized in the analyzer to suppress the speckles.

The light from the laser diode was directed onto the butt of the 3-mm diameter fiber-optic bundle. In turn, another butt of the fiber-optic bundle directed the beam onto a rotating mirror. The angle between the perpendicular to the mirror plane and the axis of rotation was 1.5°. When the mirror was rotated, the distance between the butt of the fiber-optic bundle and a microarray was changed, and the reflected beam depicted an ellipse at the plane of the microarray. Consequently, there was a blurring of the speckles in the object plane, and the illumination intensity in the FOV of the analyzer, which was averaged over the registration time, became more uniform.

Fig. 2. (A). A scheme of a device for speckle suppression. (B). Image and calculations of $I$ of a gelatin film surface that was stained with Cy5 and measured in spectral region specific to the dye. a. Image of the film surface fluorescence that was obtained when excited with a defocused laser beam and a motionless mirror. The speckles caused local variations in the illumination brightness, which significantly decreased the accuracy of the fluorescent measurements. c. Image of the same fluorescent film surface area that was obtained with a rotated mirror. b and d – Distributions of $I$ of the film surface area along the rectangular frames in Fig. 2(B) a and c. The areas inside the dotted frames in a and c represent the rectangles with a width that was equal to the diameter of one microarray cell (100 μm). The measurements were conducted in a circular surface area with a diameter of 100 μm, which was moved along the frame. $I$ was expressed in arbitrary units (a.u.) as the sum of the signal values from all the pixels that covered the measured area divided by the number of pixels. The X-axis shows the distance from the left edge of the frame; the Y-axis shows $I$ in a.u.

Images of the fluorescent gelatin surface of fine-grained Mikrat 300 photographic film stained with Cy5 and measurements of $I$ along the frames are presented in Fig. 2(B); a and b for the motionless mirror, c and d for the rotated mirror. The plots demonstrate that the device shown in Fig. 2(A) effectively suppressed the speckles that resulted from the interference of coherent laser rays on the surface.

The deviation of the signals from the average value of $I$ within the frames in Fig. 2(B) captures the effect of the speckles on the accuracy of the measurements. The coefficient of variation ($V$), which represents the standard deviation of the illuminated area brightness from the average over the entire FOV, can be expressed as follows:
\[ V = \sqrt{\frac{\sum_{i=1}^{n}(I_i - \overline{I})^2}{n}} / \overline{I} \] (1)

where \( \overline{I} \) is the average value of \( I \) within the measured area (rectangular frame) and \( n \) is the number of the measurements.

Let \( V_0 \) be the coefficient of variation of \( I \) of the area with a diameter of 100 µm within the rectangular frame before speckle suppression (the mirror is motionless) and \( V_1 \) the same value after speckle suppression (the mirror is rotated). The speckle suppression coefficient, \( A \), may be expressed by the following equation:

\[ A = (1 - V_1 / V_0) \times 100\% \] (2)

where \( A \) may vary from 0% (no speckle suppression) to 100% (complete speckle suppression).

Calculations of the value of \( I \) of the stained photographic film before and after speckle suppression according to Eq. (1) show that the variation coefficient before speckle suppression \( V_0 = 0.086 \), and after speckle suppression \( V_1 = 0.007 \). Equation (2) indicates that the speckle suppression coefficient with the device shown in Fig. 2(A) is 92%, and the variation in the excitation brightness calculated according to Eq. (1) for the area occupied by one microarray cell is less than 1%.

It should be noted that the undulation in the curve in Fig. 2(B) that depicts the variation in \( I \) of the photographic film surface is caused by both the effect of speckles and the non-uniform illumination of the analyzer’s FOV (see next section).

3.3 Accuracy measurements of \( I \) for microarray cells located in various FOV areas

To factor non-uniform brightness of the FOV caused by defocused laser illumination, a series of microarrays with cells containing the same concentration of Cy5-labeled oligonucleotide molecules was manufactured. The distance between the cell centers was 600 µm, the diameter of the cells was 100 µm, and each microarray featured 11 rows with 14 cells per row, for a total of 154 cells. The cells located close to the edges of the FOV fluoresced more weakly than those located in the center of the FOV; this variation was caused by the brighter illumination of the FOV central areas. To quantitatively measure the value \( I \) of microarray cells in different areas within the FOV while excited with a defocused laser beam, the normalizing coefficients were calculated, which accounted for the difference in the illumination brightness of these areas. It was carried out by measuring the value \( I \) of all the microarray cells and calculating that normalizing coefficients that would equalize the values of \( I \) of cells located in different parts of the FOV.

The normalizing matrix was calculated as follows. In a digitized microarray image, the pixels located in the centers of the cells were determined. Each of these pixels (14 x 11 pixels) was assigned a value equal to the average signal value of the pixels that covered the cell. For all other pixels within the microarray cell set, \( I \) was calculated based on the bilinear interpolation of the pixels that corresponded to the centers of the nearest cells. The values of \( I \) outside the set of microarray cells were calculated by extrapolation.

Because each microarray is different, there is no single microarray that can be used to calculate the normalizing matrix. First, the microarray cells may differ in size and volume. Second, the cells form a set whose position may vary in the analyzer’s FOV from one microarray to another. Third, the centers of the individual microarray cells may not be in the center of the corresponding set mesh center. An average of the pixel signal values obtained from several microarrays allows one to obtain an average surface of values for the calculation of normalizing coefficients that equalize the measurement results of the cells located in different parts of FOV.
Pixel signal values were measured for 30 normalizing microarrays that contained the same concentration of Cy5-labeled oligonucleotide molecules in the 14 x 11 cells; each sensor pixel signal was averaged, and the resulting averaged normalizing matrix was calculated as previously described. The averaged signal values for all pixels in the CCD camera served as normalizing coefficients, which allowed for the illumination brightness difference in the FOV to be taken into account.

To estimate the influence of speckle suppression of the reproducibility of the results we measured $I$ of one and the same microarray cell after repetitive removal and insertion of a microarray into the holder with still and rotated mirror. In 5 consecutive measurements deviation of value $I$ for a cell containing Cy3 from the average $I$ were 12.5% for motionless mirror, and 2.0% for rotated mirror. The values for Cy5 were 10.3% for motionless and 1.8% for rotated mirror. These results agree with the data presented in Table 1 which shows that after suppression of the speckles and normalization of the FOV a microarray cell gives practically the same value of $I$ regardless of location. Similar experiment with Cy7 was not performed as the dye faded during repetitive exposures.

To estimate the effectiveness of the normalizing coefficients and determine the accuracy of the measurements, a microarray was manufactured with one row of cells that contained the same concentration of the Cy5-labeled ATGCATGC oligonucleotide. The diameter of the cells was 100 µm, and the distance between the cell centers was 600 µm. The microarray was placed in the microarray holder such that the row was moved within the FOV along one axis, and 7 images with different locations of the microarray cells were obtained and processed. The values of $I$ of the microarray cells were expressed as previously described, namely, as the sum of the values from all pixels that covered the cell divided by the number of pixels. No bleaching was detected in the subsequent measurements, which allowed for the measurement of $I$ in the same microarray cells at a different location in the FOV. Totally, the values of $I$ of 14 microarray cells were measured in 7 locations within the FOV.

Table 1. Normalized values of $I$ for the cells of one row of a microarray at different locations in the analyzer’s FOV*

| Row | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   | 13   | 14   | δ  |
|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|----|
| 1   | 652  | 665  | 645  | 655  | 646  | 664  | 636  | 624  | 634  | 642  | 641  | 626  | 625  | 625  | 3,2 |
| 2   | 654  | 669  | 654  | 661  | 658  | 642  | 652  | 635  | 630  | 630  | 629  | 614  | 629  | 621  | 4,3 |
| 3   | 640  | 642  | 634  | 666  | 654  | 644  | 628  | 627  | 629  | 640  | 616  | 629  | 623  | 614  | 4,1 |
| 4   | 647  | 651  | 654  | 644  | 641  | 659  | 645  | 631  | 642  | 621  | 624  | 610  | 653  | 614  | 3,8 |
| 5   | 645  | 663  | 660  | 662  | 661  | 659  | 644  | 629  | 636  | 633  | 640  | 617  | 637  | 615  | 3,7 |
| 6   | 652  | 654  | 648  | 659  | 661  | 649  | 636  | 642  | 651  | 642  | 637  | 630  | 634  | 618  | 3,3 |
| 7   | 656  | 651  | 643  | 650  | 655  | 654  | 654  | 633  | 634  | 630  | 624  | 624  | 638  | 628  | 2,5 |

*The icon Nº in the left upper angle shows for lines 1 – 7 a position of a cell in the FOV at which normalized $I$ is calculated. The same icon Nº shows for the columns 1 – 14 cell numbers in the row. Values of the lower line ($Δ%) represent maximal deviation of normalized value of $I$ of an individual cell from the average value obtained at 7 different cell locations in the FOV divided by the average (in percentage). Values of the right column (%) represent maximal deviation of value $I$ of an individual cell from the average $I$ of cells in the row divided by the average in percentage.

The measurement results presented in Table 1 show that the maximum difference in $I$ between any microarray cell from a row and the average value of 7 measurements for the same cell in different parts of the FOV was less than 2.5% for any individual cell (the columns of the Table 1). These data suggest that the normalizing matrix allows for highly accurate measurements of $I$ at various locations of a cell in the FOV. The difference in $I$ between cells in the row was greater, approximately 4.3%. This value included both the error caused by the accuracy of the analyzer and that caused by the variation in cell volume during the microarray manufacturing process.
Because microarrays may fade during long-term storage and multiple measurements, they cannot be used for regular operability control of the analyzer. They are only suitable for the calculation of the normalizing coefficients during the alignment of the analyzer. To periodically check the operability of the analyzer and to ensure that the brightness or distribution of illumination of the FOV were not changed, an image of a fluorescent glass was regularly obtained and compared with the image of the same glass obtained during the normalization process. When a difference in the images does arise (laser or alignment was out of order, etc.), the normalizing coefficients should be recalculated using a new set of normalizing microarrays.

3.4 Estimation of efficiency of speckle suppression in FOV for three spectral regions

Estimation of speckle suppression for the whole analyzer FOV for three different wavelengths was calculated as follows. Images of gelatin film stained with corresponding dye were obtained with still and rotated mirrors. Coefficient of variation of $I$ of the areas covering 3x3 pixels of the image detector was measured within the whole analyzer FOV. The 3x3 pixel area was chosen due to the fact that the image detector contained “hot” and “cold” pixels giving signals that differed considerably from true values. Averaging over 9 pixels reduces the influence of these pixels to few percent. The coefficients of variation ($V$) were calculated according to Eq. (1), speckle suppression coefficients ($A$) – according to Eq. (2). Corresponding data are shown in Table 2. It might be seen that rotated mirror efficiently suppresses speckles in green and red spectral regions, and not so efficiently in IR region. It is caused by the fact that in IR region speckles are weak and more blurred; therefore the illumination of small parts of FOV even without the device for speckle suppression is more uniform.

| Wavelength | Variation coefficient ($V$) | Speckle suppression coefficient ($A$) (%) |
|------------|-----------------------------|-----------------------------------------|
|            | Motionless mirror           | Rotated mirror                          |
| 532 nm     | 0.31                        | 0.034                                   | 89           |
| 655 nm     | 0.11                        | 0.015                                   | 86           |
| 750 nm     | 0.022                       | 0.016                                   | 27           |

To estimate the influence of speckle suppression of the reproducibility of the results we measured $I$ of one and the same microarray cell after repetitive removal and insertion of a microarray into the holder with still and rotated mirror. In 5 consecutive measurements deviation of value $I$ for a cell containing Cy3 from the average $I$ were 12.5% for motionless mirror, and 2.0% for rotated mirror. The values for Cy5 were 10.3% for motionless and 1.8% for rotated mirror. These results agree with the data presented in Table 1 which shows that after suppression of the speckles and normalization of the FOV a microarray cell gives practically the same value of $I$ regardless of location. Similar experiment with Cy7 was not performed as the dye faded during repetitive exposures.

3.5 Increasing the range of measured fluorescence intensities

The dye concentration in fluorescently labeled cells within the microarray can vary by several orders of magnitude. Therefore, a microarray may contain both weak and bright fluorescent cells at any exposure time. As a result, the signals of the pixels that cover these cells may exceed the linear range of the CCD camera.
Measurements of $I$ for a fluorescent red glass showed a linear proportionality between the signal values of the CCD pixels and exposure time or illumination intensity up to 2700 a.u. According to the manufacturer’s data, the camera had a dynamic range of 12 bits (4096 a.u.); however, there was a systematic deviation from linearity at pixel values greater than 2700 a.u. because of pixel saturation effects. The camera background noise was approximately $\pm$ 20 a.u., which means that the true linear region spanned from 60 a.u. (3 times higher than the background noise) to 2700 a.u.

The linear range of fluorescent measurements may be expanded by increasing the exposure time for weak fluorescent cells, and contrary by decreasing it for bright fluorescent cells. The value $I$ of microarray cells may be calculated by the actuation of all signals to those obtained at the maximum exposure time. This procedure was performed automatically by the analyzer software.
To test the possibility of expanding the linear range of the registrations through a series of measurements with different exposure times, we obtained the microarray images shown in Fig. 3 at various exposure times. A scheme of the microarray is shown in Fig. 3(A)(a). Figures 3(A)(b), 3(A)(c) and 3(A)(d) show fluorescent images of the microarray containing three dyes (Cy3, Cy5 and Cy7 respectively) obtained in the spectral regions specific to each dye. The concentrations of the dyes in the microarray cells are presented in Fig. 3(A)e to the right of the images.

Light obtained by an image detector is proportional to the exposure time and intensity of excitation light. Divergence from the signals’ proportionality may be caused by dye fading or by non-linearity of the image detector (CCD camera). Our experiments showed that the dyes Cy3 and Cy5 are photo-stable and do not fade for exposure time up to 30 sec. The dye Cy7 is less photo-stable and fades with exposure time of about 10 sec. Measurements of cells containing different concentrations of Cy3 or Cy5 show that in the linear range of CCD camera there is a proportionality between measured $I$ and the dye concentration in the cell.

We measured the value of $I$ for all the microarray cells shown in Fig. 3(A)(c) at three exposure times: 250 msec, 1000 msec, and 4000 msec. The values of $I$ of the cells were calculated as previously described. The results are shown in the Table 3 and in Fig. 3(B).

Then, according to the expanding algorithm, the signal values obtained at 250 msec were multiplied by sixteen, those obtained at 1000 msec were multiplied by four, and those obtained at 4000 msec were multiplied by unity. These data are shown in Fig. 3(B) as black bars. The region indicated by D1 represents the linear range of the CCD camera, and the region designated D2 represents the expanded measurement range, which was achieved by obtaining images at three exposure times. The numerical values D1 and D2 are 2700 and 43000 a.u., respectively and roughly correspond to a 1:16 ratio. Thus, for the image shown in Fig. 3(A)(c), the range of quantitative $I$ measurements of microarray cells was extended by a factor of 16. The numerical values of the measurement are shown in Table 3.

### Table 3. Values of $I$ for microarray cells containing different concentrations of Cy5 at different exposure times. Image of the microarray is shown in Fig. 8c, graphic representation in Fig. 3(B). The values used for calculation of the results according to the expanding algorithm and the expanded values are given in bold. Grey cells in the columns demonstrate proportionality between $I$ and exposure time, grey cells in the lines – between $I$ and the dye concentration. Lower line shows the values of $I$ calculated according to the dye dilutions (higher value corresponds to higher dye concentration in the upper Table cell; other values are obtained by repeated division by two); $t$ – exposure time

| Cell number | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  |
|-------------|----|----|----|----|----|----|----|----|----|
| Dye concentration (M) | 8·10^{-7} | 4·10^{-7} | 2·10^{-7} | 1·10^{-7} | 5·10^{-8} | 2·5·10^{-8} | 1·3·10^{-8} | 6·10^{-8} | 3·10^{-8} |
| $t$ – 250 msec | 1530 | 785 | 407 | 199 | 113 | 46  | 28  | 35  | 22  |
| $t$ – 1000 msec | 3090 | 2990 | 1620 | 790 | 412 | 192 | 103 | 45  | 25  |
| $t$ – 4000 msec | 3130 | 3110 | 3020 | 2860 | 1782 | 759 | 405 | 215 | 105 |
| $I$ according to the expanding algorithm | 24480 | 12560 | 6512 | 3184 | 1808 | 768 | 412 | 215 | 105 |
| $I$ according to dilution data | 24480 | 12560 | 6120 | 3060 | 1530 | 765 | 382 | 191 | 96  |

The value of $I$ of a microarray cell depends on both the dye concentration in the deposited solution and the cell size; therefore, a deviation from linearity between the measured and calculated values depends on the analyzer accuracy and the reproducibility of the microarray manufacturing technique.
3.6 Analysis of the microarray cells containing mixtures of dyes

When conducting quantitative fluorescent measurements of biological molecules labeled with different fluorochromes and mixed in the same microarray cell, it is important to know whether the excitation or fluorescence spectra of the dyes used overlap.

Microarray cells containing Cy3 were not detected if the image was obtained in spectral regions specific to Cy5 (Fig. 3(C)). Similarly, microarray cells containing Cy5 were not detected in spectral regions specific to Cy3 (Fig. 3(B)), which means that relative concentrations of Cy3 and Cy5 in cells that contain a mixture of the two can be independently determined. To measure the absolute concentration of the dyes within the cells, two additional reference cells were added to the microarray, each of which contained only one dye of known concentration. The ratio between the value $I$ of the cell containing the mixture of the dyes obtained in the spectral region specific to one dye and the value $I$ of the reference cell containing the same dye yielded the desired value. In such cases, the measurement error in the dye concentrations will depend on the accuracy of the analyzer plus the variation in the volume of biochip cells that are made during the manufacturing process.

When conducting quantitative measurements of microarrays that contain two dyes with overlapping excitation and/or fluorescence spectra (e.g., Cy5 and Cy7), the value $I$ of the reference cells with only one dye of known concentration also allows for the determination of the dye concentrations in the cells that contain their mixture. The following is an example of the necessary calculations.

Let the microarray reference cells R1 and R2 contain only one dye, either Cy5 or Cy7, respectively, at known concentrations $C_{Cy5}(R1)$ and $C_{Cy7}(R2)$.

![Fig. 4. A microarray containing cells with mixtures of the dyes Cy5 and Cy7. A scheme of the microarray is shown on a. The reference cell shown in the left corner, designated R1, contained only Cy5 at a concentration of $10^{-7}$ M. The reference cell shown in the right corner, designated R2, contained only Cy7 at a concentration of $5 \times 10^{-6}$ M. Cells designated as 1, 2, 3 contained mixtures of Cy5 and Cy7 at different ratios. b – An image of the microarray obtained in the spectral regions specific to Cy5. c – An image of the microarray obtained in spectral regions specific to Cy7. Measurement results are presented in Tables 4.](image)

To determine the dye concentrations in a microarray of N cells, the values $I$ of all cells (including reference cells R1 and R2 with known dye concentrations) should be measured using the excitation and fluorescence spectral regions specific to Cy5 – $I_{Cy5}(R1)$, $I_{Cy5}(R2)$, $I_{Cy5}(1)$, $I_{Cy5}(2)$, ..., $I_{Cy5}(N)$ and to Cy7 – $I_{Cy7}(R1)$, $I_{Cy7}(R2)$, $I_{Cy7}(1)$, $I_{Cy7}(2)$, $I_{Cy7}(3)$, ..., $I_{Cy7}(N)$, where $I_{Cy5}(R1)$, $I_{Cy5}(R2)$, $I_{Cy7}(R1)$, $I_{Cy7}(R2)$ are the values of $I$ of the reference cells measured using spectral regions specific to Cy5 and Cy7, respectively, whereas $I_{Cy5}(j)$ and $I_{Cy7}(j)$ are the same values for microarray cell “j”.

The corresponding coefficients are calculated as follows:

\[
K_{Cy5} (R1) = \frac{I_{Cy5} (R1)}{C_{Cy5} (R1)}, K_{Cy5} (R2) = \frac{I_{Cy5} (R2)}{C_{Cy5} (R2)}
\]

\[
K_{Cy7} (R1) = \frac{I_{Cy7} (R1)}{C_{Cy7} (R1)}, K_{Cy7} (R2) = \frac{I_{Cy7} (R2)}{C_{Cy7} (R2)}
\]

The concentrations of Cy5 and Cy7 in any microarray cell “j”, $C_{Cy5}(j)$ and $C_{Cy7}(j)$, are calculated by solving the following system of linear equations:
The microarray containing the mixtures of Cy5 and Cy7 in the cells was manufactured to check the efficiency of the approach described above. The values of \( I \) of all microarray cells were measured using spectral regions specific to each dye. The data are presented in the Table 4.

Using the known concentrations of the dyes in cells R1 and R2 and the corresponding values of \( I \) obtained in the spectral regions specific to the dyes, the coefficients were calculated according to Eq. (3). Then, according to system of Eq. (4), the concentrations of Cy5 and Cy7 were calculated in cells 1, 2 and 3 in the middle microarray column. The deposited and calculated concentrations of the dyes in the microarray cells are presented in the Table 5. The table shows that the accuracy with which the dye concentrations in the cells were determined was within ± 5%.

### Table 4. Measured values of \( I \) of the microarray cells shown in Fig. 3

| \( I \) of the cells in spectral regions specific to Cy5 |
|------------------------------------------------------|
| \( I_{Cy5}(R1) = 1746 \) | \( I_{Cy5}(1) = 822 \) | \( I_{Cy5}(R2) = 302 \) |
| \( I_{Cy5}(2) = 1068 \) | \( I_{Cy5}(3) = 1339 \) |

| \( I \) of the cells in spectral regions specific to Cy7 |
|------------------------------------------------------|
| \( I_{Cy7}(R2) = 0 \) | \( I_{Cy7}(1) = 1020 \) | \( I_{Cy7}(R2) = 1439 \) |
| \( I_{Cy7}(2) = 762 \) | \( I_{Cy7}(3) = 503 \) |

### Table 5. Deposited and calculated concentrations of the dyes Cy5 and Cy7 in the cells shown in Fig. 3.

| Concentration of Cy5 in the cells 1, 2, 3 |
|----------------------------------------|
| Concentration in the cell R1 | Deposited concentrations | Calculated concentrations |
| \( 10^{-7} \) | \( 3.3 \times 10^{-7} \) | \( 3.5 \times 10^{-7} \) |
| \( 5 \times 10^{-8} \) | \( 5.2 \times 10^{-8} \) |
| \( 6.7 \times 10^{-8} \) | \( 7.1 \times 10^{-8} \) |

| Concentration of Cy7 in the cells 1, 2, 3 |
|----------------------------------------|
| Deposited concentrations | Calculated concentrations | Concentration in the cell R2 |
| \( 3.3 \times 10^{-8} \) | \( 3.5 \times 10^{-8} \) | \( 5 \times 10^{-8} \) |
| \( 2.5 \times 10^{-9} \) | \( 2.7 \times 10^{-9} \) |
| \( 1.7 \times 10^{-9} \) | \( 1.8 \times 10^{-9} \) |

Using the same approach, it is possible to determine the concentrations of three dyes with overlapping spectral characteristics in any cell of the microarray. In this case, the microarray would contain 3 reference cells with one individual dye at a known concentration. The value \( I \) of the microarray cells should be measured over the spectral regions specific to each dye. In this case, the concentrations of the dyes should be calculated using three linear equations with three unknown quantities.
3.7 Throughput of the analyzer

The time required to replace a microarray in the holder was approximately 15 sec. Additionally, it took approximately 30 sec to analyze a microarray containing 100 – 200 cells. The total time included the time required to automatically choose the exposure time, to obtain and process the images at different exposure times to increase the linear region of the measurements, to calculate the fluorescence intensity of each microarray cell and to output the tabular results. These procedures were performed by the software. The results suggest that when using one type of microarray, it can be analyzed approximately one microarray per minute or more than 50 microarrays per hour.

4. Discussion

The main factors that affect the accuracy with which the value of $I$ of a sample is measured when using instruments based on wide-field luminescence microscopy are the presence of speckles caused by interference of coherent beams and the non-uniformity of the FOV illumination intensity. The device used for speckle suppression, which contains a fiber-optic bundle and a rotating mirror, allows for the effective decrease of local variations in the fluorescence excitation intensity in the FOV. The signals from individual microarray cells, which are located at different positions within the analyzer’s FOV, are normalized by introducing the matrix of normalizing coefficients. In this study, the combination of a speckle suppression device and normalizing coefficients allowed for measurement accuracy as high as ± 5%.

To analyze the value $I$ of microarray cells, laser scanners are widely used. These scanners can measure the value $I$ of two-dimensional microarray cells with suitable accuracy. However, when working with three-dimensional microarray cells, which are manufactured during co-polymerization of a probe with gel monomers, it becomes necessary to take into account the fluorescence of the cell layers that do not lie within the focus of the confocal scanner; otherwise, either a systematic error can arise in the measurements or the measurement time is sharply increased and the image-processing program becomes more complicated. Analyzers that are based on wide-field luminescence microscopy measure the total value $I$ of all layers in microarray cells with suitable accuracy. The developed analyzer has significant advantages in conducting large-scale studies using the same type of microarrays and also allows for scientific studies of kinetic processes that run in parallel in different microarray cells, such as DNA-DNA, DNA-RNA, or RNA-protein interactions.

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Disclosures

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