Hyperchromatic Cytometry Principles for Cytomics Using Slide Based Cytometry

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Background: Polychromatic analysis of biological specimens has become increasingly important because of the emerging new fields of high-content and high-throughput single cell analysis for systems biology and cytomics. Combining different technologies and staining methods, multicolor analysis can be pushed forward to measure anything stainable in a cell. We term this approach hyperchromatic cytometry and present different components suitable for achieving this task. For cell analysis, slide based cytometry (SBC) technologies are ideal as, unlike flow cytometry, they are non-consumptive, i.e. the analyzed sample is fixed on the slide and can be reanalyzed following restaining of the object.

Methods and Results: We demonstrate various approaches for hyperchromatic analysis on a SBC instrument, the Laser Scanning Cytometer. The different components demonstrated here include (1) polychromatic cytometry (staining of the specimen with eight or more different fluorochromes simultaneously), (2) iterative restaining (using the same fluorochrome for restaining and subsequent reanalysis), (3) differential photobleaching (differentiating fluorochromes by their different photo-stability), (4) photoactivation (activating fluorescent nanoparticles or photocaged dyes), and (5) photodestruction (destruction of FRET dyes). Based on the ability to relocate cells that are immobilized on a microscope slide with a precision of ~1 μm, identical cells can be reanalyzed on the single cell level after manipulation steps.

Conclusion: With the intelligent combination of several different techniques, the hyperchromatic cytometry approach allows to quantify and analyze all components of relevance on the single cell level. The information gained per specimen is only limited by the number of available antibodies and sterical hindrance.

Key terms: cytome; iterative restaining; LSC; photobleaching; photodestruction; photoactivation; photoconversion

In the post-genomic era, multicolor analysis of biological specimens has become increasingly important in various fields of biology, in particular because of the emerging new fields of high-content and high-throughput single cell analysis for systems biology (1–6) and cytomics (7,8). Areas of research and diagnosis with the demand to virtually measure “anything” in the cell include immunophenotyping, rare cell detection, and characterization in the case of stem cells and residual tumor cells, tissue analysis, and drug discovery. Often only small sample volumes are available or samples are very precious. In these cases, multicolor analysis enables ideally to determine all cellular constituents of interest in a single analytical run.

The development of fluorescent dyes of organic and inorganic origin in the recent years greatly facilitated multicolor approach. Fluorochromes emitting in the near infrared, like tandem dyes (PE-Cy7, APC-Cy7) (9–11), as well as fluorescent nanoparticles or quantum dots (QDs) (12,13) offer the opportunity to increase the number of simultaneously measurable fluorochromes. These developments initially led to polychromatic cytometry, i.e., the ability to perform up to 17-color immunophenotyping by three laser flow cytometry (FCM) (12) or eight-color immunophenotyping using a dual laser equipped Laser Scanning Cytometer (LSC) (14), a Slide Based Cytometry (SBC) instrument. These approaches revealed the existence of new cell phenotypes with discrete combinations of antigen expression or cytokine production patterns. Our group has demonstrated that differentiation between Cy5 and Cy5.5 tandem conjugates with close emission spectra is possible via optical filter change and subsequent remeasurement. This method allows to simultaneously analyze eight fluorochromes (FITC, PE, PE-Cy5, PE-Cy5.5, PE-Cy7, ...
measurable in a single run is limited. Finally, FCM systems in the cell sorting or with specialized imaging of each individual cell's morphology is only possible to measure the identical cell repeatedly. In addition, the analysis of time consuming cell sorting or with specialized imaging of each individual cell's morphology is only possible to measure the identical cell repeatedly.

Furthermore, the precise storage of the location of each measured event on the slide allows reanalysis after modifications of the specimen, e.g., restaining, or changes of instrument settings. Both analyses e.g., before and after restaining, can then be fused to one data file (a feature of the LSC software called “merging”). This approach has been used to reanalyze cell nuclei after immunophenotyping (16), to combine alive and dead information (17), to analyze destaining kinetics (18), and to reanalyze surface antigens with second and third sets of antibodies (19). A specific feature of SBC that cannot be covered by FCM is the quantitative analysis of tissue sections. Steiner et al. (20) demonstrated that immunophenotyping of leukocytes in tissue sections is possible with confocal microscopy. Gerstner et al. (21) showed three- and four-color immunophenotyping of tonsil sections, Lenz et al. (22) established quantitative two- and three-dimensional analysis of the distribution of nuclei and neurons in brain tissue sections by LSC. Recently, approaches have been proposed towards the concept of 3D-cytometry of tissue sections and in tissue cultures termed Tissomics (23–25).

Polychromatic cytometry of cells by fluorescence microscopy is of special importance in two different settings: First, in patients with low blood volume such as neonates and in critically ill infants for whom every effort has to be taken to reduce the blood volume needed for diagnostics. This, for instance, can be achieved by increasing the amount of analyzed antigens (i.e. CD or activation markers) per sample. Second, since our view of cellular systems gets more complex, the characterization of cell subsets makes the use of more parameters necessary and even unavoidable (11,12,14). Therefore, highly multiplexed characterization of cells on the single cell level is a pivotal technology for single cell genomics, proteomics, and cytometrics (1,2,4,7,8,26–28). With the increased number of measurable characteristics of a cell in addition with its morphological evaluation, SBC multiplexed cell analysis is a powerful analytical and diagnostic tool. Its most important impact can be expected in drug discovery in the pharmaceutical environment (e.g. tissues, cell cultures).

In our view, SBC instruments are the ones best suited to perform hyperchromatic analysis. Although FCM instrumentation equipped with a multitude of lasers and applying the plethora of presently available fluorescent dyes for labeling is able to simultaneously measure 17 or probably even more colors (12), it is hampered by its inability to measure the identical cell repeatedly. In addition, the analysis of each individual cell's morphology is only possible after time consuming cell sorting or with specialized imaging flow cytometers (33) wherein the number of colors measurable in a single run is limited. Finally, FCM systems

**Fig. 1.** Schematic hyperchromatic cytometry. The scheme summarizes the different components of hyperchromatic cytometry and how these components can be combined sequentially. At the end of one cycle and following iterative restaining new analytical cycles can follow. The combination of these methods enables the step from polychromatic to hyperchromatic cytometry. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

are by principle unable to analyze cells in their natural environment (e.g. tissues, cell cultures).

In the following, we will brief on SBC technology and then review various components of measurement or labeling that are suitable for performing hyperchromatic cytometry (Figs. 1 and 2), starting with the established technique of polychromatic cytometry. We will primarily emphasize those components of hyperchromatic cytometry that have been tested in our laboratory. In the last chapter, the application areas and usefulness of additional various components will be discussed.

**SLIDE BASED POLYCHROMATIC CYTOMETRY**

**Principle**

SBC with fluorescence microscope based instruments is an analytical technique that allows rapid quantitative analysis of a high number of individual cells in suspension, in culture, or in tissue sections tagged with fluorescent dyes and immobilized on a slide. Today, several SBC systems are commercially available or under development. Some of them rely on lasers (23,34,35) others on Arc-lamps as excitation light sources (36,37).

In the following, we will demonstrate the feasibility of the components for hyperchromatic cytometry, using the first commercially available SBC instrument, the LSC. The LSC and its function is detailed elsewhere (23,38–40). LSC

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FIG. 2. Components of hyperchromatic cytometry. This figure shows schematically how the different components of hyperchromatic cytometry outlined in Figure 1 work. (1) Analysis with different filter settings. Samples stained with fluorochromes with similar emission spectra (e.g. PE-Cy5 and PE-Cy5.5) cannot be distinguished with one set of filters. Using different filters and subsequent merging of both analyses enables their discrimination. (A) Schematically stained cells seen by a filter optimal for detection of PE-Cy5. (B) The same cells with a “Cy5.5-filter”. (C) By merging both analyses a combined data file results with the fluorescence information from both filter sets. Also double positive cells can be detected (e.g. Fig. 3). (2) Iterative restaining. The sample is stained repeatedly with different antibodies conjugated to the same fluorochrome. (A) The first staining of a sample, (B) same sample restained with the same fluorochrome but conjugated to another antibody, and (C) third staining with the same fluorochrome and yet another antibody. In a merged file, these cells can be displayed before vs. after iterative restaining. The new emerged fluorescence shifts off the diagonal and can be identified as a new population (e.g. Fig. 4). Cells are labeled with arrows that receive an additional staining in the subsequent staining step, i.e., cell in A (arrow) is in B double labeled with two different antibodies, cell in C (left arrow) is triple labeled in C. (3) Differential photobleaching. Fluorochromes differ in their photostability (Fig. 5). These differences allow to combine fluorochromes with different photostability. Sensitive fluorochromes can be bleached, e.g. by laser light, and the residual fluorescence belongs to the photostable fluorochrome. In a file that combines the data before and after bleaching, both fluorochromes can be distinguished unequivocally. Arrow marked cells demonstrate cells labeled with photosensitive fluorochromes (e.g. Fig. 6). (4) Photoactivation. Specific fluorescent dyes such as QDs can be activated to fluoresce by light. Initial measurements generate only weak signals (A) but after repeated scanning with the laser the fluorescence signals increase (B). (5) Photodestruction. Tandem dyes that rely on fluorescence resonance energy transfer (FRET) produce in most cases a fluorescence signal not only in the acceptor (A) but also in the donor channel (B). By exposure to light, the FRET is destroyed so that the acceptor fluorescence disappears (C), whereas that of the donor dye increases (D). Photodestruction of FRET can help to discriminate between FRET and non-FRET fluorescence. (e.g. Fig. 7). (6) Photoconversion. By direct exposure to light the color of certain fluorescent proteins can be switched, e.g., the protein KAEDE turns its emission after UV illumination from green to red. (7) Spectral fingerprinting. A completely different approach for hyperchromatic cytometry demonstrates spectral fingerprinting where the entire fluorescence spectrum of the cell is detected.
like other flow and slide based technologies measures stoichiometrically expression levels of multiple cellular components simultaneously. The LSC setup is built around a conventional epi-fluorescence microscope. Fluorescence is excited by laser light that is reflected by mirrors onto the specimen. The laser sources used are an Argon laser for blue light (488 nm) excitation and a red light emitting Helium–Neon laser (633 nm). Optionally, also a violet (405 nm) or a green laser (543 nm) can be included. The slide is scanned by the laser light and the emitted fluorescence is collected by up to four photomultiplier tubes (PMTs) each detecting light of a certain bandwidth defined by optical filters. Additionally, the light scattered by the objects on the slide is detected by a photodiode. The measured signals of a scanned field are reconstructed to pixel-per-pixel-maps (i.e. images) for all fluorescence or scatter channels. These images are used to define the objects of interest and to acquire cytometric data on a single-cell basis in the various channels. After one field has been analyzed by the software, the microscope stage moves to the next field to be scanned, performs the next analysis and so on, until the whole scan area has been analyzed.

The system produces data similar to FCM in listmode 3.0 format, with the unique feature that also the x- and y-coordinates of the cells’ position are stored with the fluorescence and forward scatter data; based on this feature, cells can be relocalized any time after the measurement. The advantage of this relocalization feature is obvious, for example, the same specimen can be measured several times with different instrument setups or after restaining with new labels. These separate measurements can be brought together into a single data file by a calculation process named “merging”. This merging process creates a virtual data file where all cells with identical x-y-coordinates in the different analyses are identified as being the same cell. With this feature, the information density gained for each cell is increased. This approach has been used by us to restain slides after immunophenotyping by a conventional morphological hematoxylin-eosin stain and to relocalize single cells for morphological analysis in solid tumors (41), fine needle aspirate biopsies (42), cytological swabs (43), and peripheral blood leukocytes (44).

**LSC Settings**

Instrumentation and software of the LSC is explained in detail elsewhere (38–40). In the standard instrumentation, the following bandpass filters are used: 530/DF30 (green), 580/DF30 (orange), 625/DF28 (red), and a 650 nm long pass filter (far red). For the detection of Cy5.5 and Cy7 tandems, filter cubes in position No. 3 and No. 4 were replaced as described in detail by Mittag et al. (14). In brief, filter configurations of the LSC for the detection of these dyes are 530/30 nm (No. 1, green), 580/30 nm (No. 2, orange), 670/20 nm (No. 3, red, detection of PE-Cy5/APC), 710/20 nm (No. 3, red, detection of PE-Cy5.5/APC-Cy5.5), and 810/90 nm (No. 4, far red). This filter configuration enables simultaneous measurement of eight different fluorochromes.

**Analysis with Different Filter Settings—Polychromatic SBC**

Originally, the filter settings of the LSC were adapted for the detection and differentiation of up to five different fluorochromes in a single run. With slightly modified filter configurations, adapted for the detection of Cy7 tandems, six different fluorochromes per analysis can be analyzed on a 2-laser LSC (FITC, PE, PE-Cy5, PE-Cy7, APC, APC-Cy7) (45). Distinguishing more fluorochromes is not possible.

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**Fig. 3.** Analysis with different filter settings. (A) Human peripheral blood leukocytes (PBL) were stained with CD14-PE-Cy5 and CD4-PE-Cy5.5. A differentiation of these fluorochromes emitting in a similar spectrum is possible because of the merge feature of the LSC. The 1st measurement was performed with a filter suitable for the detection of PE-Cy5. For the 2nd measurement a “PE-Cy5.5” filter was used. In the merged file, one can discriminate both fluorochromes in a dotplot displaying first vs. second measurement. (B) The same sample was stained with three fluorochromes with similar emission spectra (PETR, PE-Cy5, PE-Cy5.5). Three measurements were performed, each one with a different filter suitable for one of the fluorochromes. By gating for PE TR fluorescence and excluding the PE-Cy5 fluorescence (left dotplot) the PE-Cy5.5 and PE TR stained cells can be differentiated in a second dotplot (right). All figures show untagged data, measurement was triggered on forward scatter and cell size >20 μm ID. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
with a single scan because of the limited number of PMTs and spectral overlap of dyes with close-by emission spectra.

With two new tandem dyes, i.e. PE-Cy5.5 and APC-Cy5.5 (46), with an emission spectrum between Cy5 and Cy7, fluorochrome number could be increased. Emission spectra of the Cy5.5 conjugates are very similar to those of their Cy5 equivalents. Those colors would normally not be distinguishable with the standard filter settings of the LSC. With different filters and subsequent remeasurement, however, discrimination of Cy5 and Cy5.5 are possible by LSC (Fig. 3A). This method is used for eight color immunophenotyping (14) by adding PE-Cy5.5 and APC-Cy5.5 to the original six color panel (37). To this end, a blood sample is simultaneously stained with all eight fluorochromes conjugated to different antibodies. The first measurement is performed using a filter suitable for both PE-Cy5 or APC fluorescences (670/20 nm); with these filter settings it is not possible to distinguish between Cy5 and Cy5.5 conjugated antibodies. For the second measurement instead a filter optimized for the detection of Cy5.5 tandems (710/20 nm) is used and the identical sample is measured again. All other instrument settings except the filter change are kept identically throughout both measurements. Finally, to combine the data of both measurements, both the *.fcs data files are merged by the software (Fig. 2.1). The technique of subsequent filter changing from polychromatic cytometry can be included to further increase fluorescence number.

With filter changing other fluorochromes can also be added, without major modifications to the instrument, e.g. PE-Texas Red (Fig. 3B) for nine-color polychromatic SBC. To this end, either a third measurement can be performed with a bandpass filter suitable for the detection of this fluorochrome (625/28 nm) or the second measurement can be performed with the “PE-TexasRed filter” in position No. 2 and the “Cy5.5 filter” in position No. 3 in order to reduce the time needed for analysis.

METHODOLOGICAL APPROACHES TOWARDS HYPERCHROMATIC CYTOMETRY

Although eight to nine colors can be used for the simultaneous quantitation of cellular components and functions (14), this number of parameters is far too low for unraveling the cytome (26,28). Because of its limitations (in general 4 PMTs), a multicolor analysis by LSC with a single scan is difficult. The major advantage of any SBC system is that the samples are not lost after analysis. This allows reanalyzing the same specimen, resulting in two or more measurements of the identical specimen. These different measurements can be combined based on the x-y-positions of every measured event. A new, merged *.fcs data file is generated with the fluorescence information for each cell from both measurements. These data can be displayed as separate parameters, i.e. virtual colors. There are several ways to exploit the relocation and merge feature in order to generate virtual colors, and we termed the combination of these different methods hyperchromatic SBC (a scheme of the hyperchromatic cytometry concept is shown in Fig. 1). In the following sections, the various components of hyperchromatic SBC are described in detail. Figure 2 shows schematically the different components for hyperchromatic cytometry.

Different Antibodies with Identical Dyes—Iterative Restaining

For substantially increasing the density of information obtained from a sample, iterative restaining can be performed (19). Iterative restaining has already been included by Schubert and coworkers into their location toponomics analysis, a highly multiplexed analysis of tissues (47,48), and was termed iterative fluorescence tagging. For this approach, a 1st staining with directly labeled antibodies and a 1st analysis is performed. Then a 2nd staining with different antibodies labeled with the same fluorochromes and a subsequent 2nd analysis is done. These steps can be repeated (i.e. iterative restaining) for a 3rd staining with a 3rd analysis and so on (Fig. 2.2). The files of the analyses are merged. In this merged data file, the identical colors appear as different parameters according to the respective measurement/analysis (e.g. “PE 1st staining” and “PE 2nd staining”). For data display, the intensity of a dye in the 1st staining is plotted against its intensity in the 2nd staining (Fig. 4); cells with unchanged fluorescence in the 1st and the 2nd staining will line up along the diagonal whereas the CD4+ cells shift off the diagonal because of their newly labeled 2nd staining (arrow).
FCM. The advantage is that the need to compensate for fluorescence spill-over is reduced, since only three or four fluorochromes are used; compensation is far more complicated when running polychromatic assays (49). By iterative restaining, experimental protocols can be improved in that each cell serves as its unlabelled control, thereby improving the detection sensitivity. In addition, a bleaching step can be included before each restaining to further improve sensitivity (47,48).

ALEXA Dyes—Differential Photobleaching

As introduced by Panchuk-Voloshina et al. (50), the series of so called ALEXA dyes have emission spectra similar to commonly used fluorochromes. However, ALEXA dyes and their conjugates are more photostable than their conventional analogs (Fig. 5). In SBC these differences in photostability can be exploited as additional parameters for increasing the simultaneously measurable fluorochromes (51). To this end, the sample is stained together with conventional fluorochromes such as FITC, PE, or APC, and analog ALEXA dyes (ALEXA488, ALEXA532, ALEXA647). The sample is then analyzed using the appropriate filters. This 1st measurement does not allow discrimination between conventional and ALEXA dyes. As a next step, the sample is photobleached by repeated scanning with the laser. Because of the differences in the photostability, this step will differentially bleach the conventional dyes but the ALEXA dyes will remain almost unchanged. After photobleaching, the sample is measured for a 2nd time (Fig. 2.3). The pre- and post-bleaching data are merged into a new .fcs-file. Per channel a dotplot is created displaying the respective fluorescence before versus after photobleaching. Since ALEXA-stained cells keep their signal unchanged or are only moderately affected by photobleaching, these cells will line up in a diagonal; cells stained with conventional dyes, however, lost their signal by photobleaching and therefore drop off the diagonal. This is exemplified for the pair of PE vs. ALEXA 532 in Figure 6.

Quantum Dots—Photoactivation

In the previous years, fluorescent nanoparticles or QDs that are based on semiconductor technology became of eminent importance for staining of biological material (52–54).
QDs are of high relevance to polychromatic analysis as on the one hand their fluorescence exhibits an eminent Stokes shift (excitation maximum in the UV but emission up to near IR depending on the QD type) and on the other hand they can be produced in many different colors (the QD color is directly correlated with their size). Their high Stokes shift allows measuring the specific fluorescence far away from the cells autofluorescence signal and without interference from organic fluorochromes. The fluorescence emission spectra have a Gaussian shape. This property reduces spill-over between near-by colors and allows the use of tighter and more specific bandpass filters in front of the PMTs. Presently, about 10 different QD color flavors are commercially available ranging from violet to near IR. This number could be substantially increased by producing particles of very homogeneous size, thus much smaller bandwidth of fluorescence emission.

There is an additional special feature of Qdots that makes them of particular relevance to hyperchromatic cytometry by offering an additional possibility to generate virtual colors: QD fluorescence can be photoactivated. We observed with QDs that by an initial scan, only a very weak emission could be excited by the Argon-laser (Fig. 7A, black line). However, after repeatedly scanning the same sample with the Argon-laser, a substantial increase of the fluorescence intensity was detected in that subset of cells that was labeled with the antibody (scheme in Fig. 2.4, example in Figure 7A, gray line and dotplots in Fig. 7B). This phenomenon, termed photoactivation, was observed by us and others (55,56). Both components of hyperchromatic cytometry, differential photobleaching, and photoactivation can be implemented simultaneously to generate virtual colors. To this end, a sample is stained with antibodies conjugated to sets of three fluorochromes of similar spectra (e.g. APC, ALEXA647, and QD655). Analysis is identical to that described for differential photobleaching. The first analysis before photobleaching and photoactivation and the second analysis afterwards are merged to one file. Again, per channel, a dot plot is generated displaying the fluorescence before versus after photobleaching and photoactivation. In this plot, cells with unchanged signal (i.e. ALEXA stained cells) will line up in a diagonal, cells with bleached signal (conventional dyes) will drop off the diagonal, and cells with activated fluorescence (QD stained cells) will shift off the diagonal. This allows detecting and differentiating three fluorochromes with similar spectra in one channel, yielding three virtual colors.

Utilization of Tandem Dyes—Photodestruction

Tandem dyes are a formation of two different fluorochromes in very close proximity (<10 nm). Between these fluorochromes a fluorescence resonance energy transfer (FRET) from the donor dye exited by the laser to an appropriate acceptor dye occurs; as a result the emission of the donor serves as excitation of the acceptor. This transfer can be destroyed by repeated excitation with laser or UV-light. Especially, Cy7 tandems are very susceptible to light. Photodestruction of FRET results in an increased fluorescence emission of the donor and a decreased fluorescence emission of the acceptor (Fig. 2.5). This can be exploited to obtain virtual colors: A sample is stained with a tandem dye. In the first measurement, not only the fluorescence of the acceptor dye (e.g. Cy7) is detectable but to some extent also the fluorescence of the donor dye (e.g. PE). After photodestruction of FRET, the sample is analyzed again and the two .fcs-files are merged. In two displays plotting the fluorescence of the donor and acceptor, respectively, before versus after photodestruction cells stained with FRET-dyes will exhibit loss of acceptor emission and gain of donor emission (Fig. 8).

Fig. 7. QDs—photoactivation. In this experiment, peripheral blood leukocytes were labeled with antiCD3-biotin followed by labeling the biotin with Streptavidin QD 605. This should label all T-lymphocytes that make about 30% of all cells. However, excitation with the Argon laser (488 nm, 10 mW) leads to a very weak signal (black curve in A; left dotplot in B) and the labeled T-cells are hardly detectable. The fluorescence intensity increases following repeated excitation with the same laser. After 15 remeasurements, the QD labeled cells become brighter and a small population of cells is now visible (dotplot in B right, population to the right of the gray curve in A). Note: The residual unlabelled cells become slightly dimmer, probably due to bleaching of autofluorescence. QDs are excitable in the blue range, but their optimal absorption is in the UV range. Therefore, photoactivation seems to be more efficient in the UV as also preliminary experiments from our lab suggest. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
Experimental Dyes—Photoconversion and Photocaging

Recent research has led to the development of fluorescent proteins that change their conformation upon excitation with light of a given wavelength. This phenomenon called photoconversion is exemplified by the protein KAEDE (57); its emission turns from green to red after UV illumination (Fig. 2.6). Although initially developed for the study of living cells, it could be exploited to generate virtual colors for multicolor immunophenotyping. The same might apply for the technique of photocaging (58–60); in this maneuver a fluorochrome is “caged” in a substance that quenches its emission. However, the caging substance is itself photosensible and can be destroyed by illumination. This can be used to free the fluorochrome that previously was hidden.

Spectral Fingerprinting

A completely different approach could be achieved by detecting the entire fluorescence spectra instead of separated spectral windows. To perform this type of analysis, PMTs must be replaced by a spectrometer. The information obtained would then consist of the entire fluorescence spectrum of each cell (Fig. 2.7). This can be achieved by a standard confocal laser scanning microscope where the pinholes are opened so that it is running in a non focal mode, i.e. depth of focus should be around 30–50 μm to collect whole cell, cytometric information (35). An alternative approach could be to replace optical filters by acoustic optical tunable filters (AOTF) that allows changing the property of the detected light by the PMTs within subseconds (61).

Including Structural Resolution—Location Proteomics, Toponomics

The aim of cytometric analysis is to collect stoichiometric information of cell constituents based on the fluorescence intensity of labeled reporter molecules. Therefore, in SBC, samples are measured with high speed, relatively low resolution and high focal depth to avoid photobleaching that hampers quantitative analysis and for increased throughput. However, the stained objects also contain structural information that is of importance to understand relationships of cells within a tissue and of molecules inside and outside a cell. This information can be obtained by going to higher resolutions and for example include confocal microscopic analysis. Higher resolution images may need longer exposure times and higher excitation power and have lower focal depth, so that confocal analysis (unless the depth of focus is increased to the cells hight) is not strictly cytometric. Therefore, the samples should be analyzed first in a cytometric setup and then reanalyzed with high structural resolution. In the end, both sets of information need to be merged on a cell-by-cell basis by appropriate software solutions.

Several other groups have started to take morphology into account for systems biology and cytomics. Murphy and coworkers (62,63) use the distribution of molecules within the cell for automated cataloguing of cell pheno-types and activity states. On the basis of 2D and 3D images, they developed hierarchical trees of structural relations (Location Proteomics) by which the software automatically recognizes and classifies different molecules in different states of the cells development. Schubert (47,48) presented a method to monitor and catalogue the relationship of dozens of antigens in the identical specimen for their MELK (Multi Epitope Ligand “Kartographie”) technology. To automatically and unequivocally recognize individual cells within tissues — a task still difficult to be performed by image analysis — different technological approaches are applied, including machine vision (25,64), tissue cytometry (65), object-oriented image analysis for high content screening (66) and on the LSC multiple thresholding (21) and phantom contouring (67). Finally, monitoring and cataloguing of multicellular arrangements and 3D interrelationship of cells in tissues or even in living objects by concepts like Tissomics (24,64) is the last challenge for quantitative analysis.

DISCUSSION

Hyperchromatic cytometry is a novel analytical approach intended to significantly increase the depth of information gained on the basis of individual cells by the intelligent combination of different analytical and preparation steps (Figs. 1 and 2). In this paper we have presented different components of the method that can be combined appropriately to achieve this highly multiplexed cell analysis. The ideal instrument for hyperchromatic cytometry is the fluorescent microscope with an imaging system that enables for SBC analysis and repositioning capabilities. Identical cells can be reanalyzed for getting more (virtual) colors, and on the other hand an in-depth view of the detailed morphology of every individual cell can be obtained.

FCM can distinguish more fluorochromes in a single analysis than any SBC instrument. As published by Perfetto et al. in 2004 (12), it allows nowadays to differentiate up to 17 colors and two light scatter signals per measurement. This result is achieved by the use of three lasers (an additional UV laser is applied) for excitation. SBC seemed still far away from these possibilities: the highest number of fluorochromes that can be differentiated with SBC was recently increased to eight (14) in an SBC instrument and also to 8 with a confocal microscope combined with spectral deconvolution (35,65). Up to now this was the highest number of fluorochromes that could be used for immunophenotyping in a quantitative fluorescence microscopic assay.

By hyperchromatic cytometry, however, virtually anything in one cell becomes measurable.

Polychromatic cytometry (14) is the basis of hyperchromatic cytometry and can be combined with one or more of the other components like iterative restaining, differential photobleaching, photoactivation, photodestruction, photoconversion, and structural analysis. Special features of each of these components are outlined below. Careful calibration and standardization (see also below) needs to be performed while setting up these types of analysis.

Iterative restaining is an elegant way to measure subsequently a high number of antigens in the identical speci-
FIG. 8. Tandem dyes—photodestruction. Some tandem dyes are very susceptible to light. The FRET from the donor to the acceptor dye is destroyed by several excitations with a laser. This results in a changed ratio of donor and acceptor fluorescence as shown in the following. (A) Cy7 fluorescence (acceptor) is decreased after six measurements, whereas the fluorescence of the donor (PE) is increased. (B) Merged file of measurement 1 and 6 of a sample stained with CD4-PE-Cy7: the Cy7 fluorescence is barely detectable after six measurements whereas a strong PE signal has appeared. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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Photobleaching can also be easily combined with photoexisting to virtually all standard organic fluorochromes. ALEXA or Cy analogues, with higher photosensitivity, are conveniently combined with polychromatic cytometry as possible for SBC (51). Differential photobleaching is very convenient by combinations of polychromatic staining and microscopic imaging to combine analysis of two dyes with similar emission spectra (FITC with ALEXA488) (50). We were the first to demonstrate that this approach is also feasible for Toponomics (19) (unbleached) PE signal to yield the PE-only fluorescence intensity. 

Differential photobleaching, photoactivation, photodestruction, and photoconversion

The beauty of all methods where fluorochromes are modified by light is that no mechanical manipulation is included. This method is not only technically simpler to perform but also non-adherent cells can be easily analyzed hyperspectrally by combinations of polychromatic staining and methods manipulating fluorochromes with light. Differential photobleaching has been first applied in microscopic imaging to combine analysis of two dyes with similar emission spectra (FITC with ALEXA488) (50). We were the first to demonstrate that this approach is also feasible for SBC (51). Differential photobleaching is very conveniently combined with polychromatic cytometry as ALEXA or Cy analogues, with higher photosensitivity existing to virtually all standard organic fluorochromes. Photobleaching can also be easily combined with photoactivation and photoconversion, as in the latter, fluorescence does either not change or increase.

Photoactivation of QDs has several important aspects for hyperchromatic cytometry as detailed in the methodology. Background fluorescence is virtually absent because high Stoke's shift and QDs are virtually unbleachable. The tendency to blink in an irregular fashion at least of some types of QDs (13) may hamper quantitation of fluorescence intensity and requires careful testing. Another aspect of QDs is probably more critical for hyperchromatic analysis. We have observed that excitation of QDs for a long period of time not only increases their brightness but also induces a shift of the emission to shorter wavelengths. The exact reason for this behavior is unclear and may be due to destruction of the outer shell of the particle. Again, careful testing is required when setting up an experimental protocol.

Photo destruction is useful for distinguishing signals of FRET dyes from those of other conventional fluorochromes. Although the latter will get dimmer, for FRET dyes the fluorescence intensity ratio of donor to acceptor will increase. Thereby a signal of a PE labeled antibody can be distinguished from the PE signal of a PE-Cy5, PE-Cy5.5, or PE-Cy7 labeled antibody. This signal can be subtracted from the original (unbleached) PE signal to yield the PE-only fluorescence intensity.

Photoconversion like photoactivation have both the enchanting properties that signals are virtually invisible before conversion or activation takes place. Because after photocconversion conventional organic dyes fluoresce the problems discussed for QDs will not apply. A photoconversion step can be applied after the original fluorescence from the organic dyes was bleached (and documented) so that the photoconverted signal appears in a background free environment.

By spectral fingerprinting one gets rid of the uncertainty as to what extent a given dye spills over into the emission signal from another dye. This approach was successfully applied by Ecker and Steiner (65) who were able to distinguish up to eight different colors in tissue sections. However, there is also an advantage in using different PMTs for the different colors. In many spectral imaging microscopes the fluorescence spectrum is detected by an array of PMTs that are set to the same sensitivity. If now a bright signal from one cell constituent is measured in parallel with a dim signal from another, the first may be out of range if we want to detect the second or vice versa. With multiple separate PMTs, the sensitivity of each of them can be set to fit the brightness of the signal in the cell.

Calibration and standardization are key issues and special care needs to be taken for them in hyperchromatic cytometry. By principle, all (optical) manipulations that affect the fluorescence emission are contradictory to cytometric analysis because the measured fluorescence intensities will be modified and will not strictly represent antigen expression levels. When samples are iteratively restained, those antigens that are stained in later steps may artificially present reduced fluorescence intensity (19). This is either due to loss of antigen availability, photodamage of the antigen, or other reasons. Therefore, we recommend using the fluo-
rescence intensity signals before further manipulation to quantify expression levels. The signals detected after manipulation serve as phenotypic markers in order to pin-point to which sub-type the cells belong.

With some additional effort, however, these signals may be standardized to yield cytometric data. To this end, one can apply control cells of known antigen expression that are treated identically to the unknown specimen. For example, samples from healthy individuals can be used for the calibration of antigen expression levels on human leukocytes (68). Alternatively, all antigen expressions are tested in a permutating order within the flow of the hyperchromatic staining and changes are precisely recorded (i.e. without manipulation vs. after manipulation). These data serve for each antigen as a calibration data base that then can be used to recalibrate fluorescence intensities. Essential is that all preparation and manipulation steps are strictly standardized and reproducible. This demand could be fulfilled by automating the whole process of hyperchromatic preparation steps.

One can expect that there will be a natural upper limit of the number of parameters that can be measured in a cell. This is not so much due to the technical limitations of the instruments but to sterical hindrance of the reporter molecules that are placed into the cell. Using non-saturating concentrations of antibodies or other markers can help to avoid this problem (48), but it has to be taken into account that fluorescence intensities may be affected. It would be useful for iterative restaining to remove all previous markers from the cell before the staining step. But this will need the use of proteases or detergents with the unpredictable side-effect of destructing antigens of interest.

Another possible problem that could occur in hyperchromatic cytometry and may need careful analysis is the phenomenon of unwanted FRET in the case that two different molecules are located very close to each other. If the expression level of each of the molecules should be recorded this is clearly not desired. Therefore, careful experiments have to be done to rule out fluorescence quenching (or enhancement) by FRET.

Repositioning of the objects after manipulation with a precision of ±1 μm is sufficient if measurements are performed in a low resolution as is the case with the LSC. Here, the major issue is that cells from two measurements are identified as being identical based on their position in an area of about 5 μm. If, however, a high structural resolution is required as in the case of toponomics or location proteomics, a highly precise pixel based recognition of identical positions from different images of the same object is crucial. Because of imprecision of motorized microscope stages errors in repositioning will naturally accumulate the more often we reanalyze the sample. This will even become worse if we have to remove the specimen from the stage for staining and then replace it again. Obviously, intelligent software solutions are necessary to unequivocally combine the different images acquired by hyperchromatic cytometry.

The advantages of SBC systems for high-content cytomtics analysis by hyperchromatic cytometry are evident. The cells of interest remain immobilized on the slide. This on the one hand is pivotal to make hyperchromatic detection possible at all. On the other hand, relocation is important for the visual inspection of cells of interest and their morphological documentation. This forms an input for the automated high-resolution structural analysis of the specimen. Structural analysis requires high spatial resolution but only to a lesser extent unperturbed fluorescence intensity. Therefore, cytometric data should be obtained first, followed by structural analysis. The combination of both cytometric and quantitative structural data tremendously boosts the information gained per cell.

To perform hyperchromatic cytometry as a routine technique in future, new instruments and new computational solutions are required. Instruments have to be developed that enable automatize staining, analysis, and photo-manipulation. Presently, several slide based instruments are under development that can at least partially fulfill this demand [e.g. 34]. New fluorochromes that have very specific features (e.g. photoconversion at very specific wavelengths) need to be developed to more specifically modify fluorences. New detection molecules smaller in size such as RNA aptamers (69) are essential to reduce sterical hindrance. Finally, solutions for combining, analyzing, and documenting the tremendous amount of data collected by hyperchromatic analysis have to be found [examples approaching this point in Refs. 48,62,70].

The possibility to analyze genomic and proteomic properties of whole cell populations on the single cell level in their natural environment is important to unravel the complexity of cells and cell systems. It opens the way to better understand complex processes in health and disease and could be an important tool for predictive and preventative medicine. Cytomics and systems biology can show information of the present status and diagnosis, and consequently allow an individualized therapy as a general practice of medicine (2,4,7,31,32,71). Furthermore, with hyperchromatic cytometry novel cell types might be recognized not detectable with presently available methods. With the proposed methods in theory, the amount of detectable cell characteristics is only limited by sterical hindrance. Hyperchromatic cytometry is, therefore, conceived as a joint cross-disciplinary effort for cytomtics, systems biology, and high-throughput-oriented research for basic, clinical, and industry scientists [1–4,7,8,26,30–32].

**LITERATURE CITED**


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