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TECHNICAL NOTE

Cytometry PART A
Journal of the International Society for Advancement of Cytometry

Automated Sample Area Definition for High-Throughput Microscopy

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Additional Supporting Information may be found in the online version of this article.

Received 8 December 2010; Accepted 24 January 2011

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Published online 15 March 2011 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.21034

Abstract

High-throughput screening platforms based on epifluorescence microscopy are powerful tools in a variety of scientific fields. Although some applications are based on imaging geometrically defined samples such as microtiter plates, multiwell slides, or spotted gene arrays, others need to cope with inhomogeneously located samples on glass slides. The analysis of microbial communities in aquatic systems by sample filtration on membrane filters followed by multiple fluorescent staining, or the investigation of tissue sections are examples. Therefore, we developed a strategy for flexible and fast definition of sample locations by the acquisition of whole slide overview images and automated sample recognition by image analysis. Our approach was tested on different microscopes and the computer programs are freely available (http://www.technobiology.ch). © 2011 International Society for Advancement of Cytometry

Key terms

high-throughput microscopy; shape recognition; sample localization; fluorescence in situ hybridization; microbial ecology

Introduction

High-throughput imaging systems became a powerful tool to address questions in a vast variety of scientific fields, such as drug discovery (1,2), phenotyping (3), or analysis of microbial communities in environmental habitats (4,5) (Zeder, unpublished data), to name a few examples. Although the specimens and the microscopic setups may be different, all microscopic screening platforms feature autonomous acquisition of digital images of multiple samples. A first and crucial step in the imaging process is the spatial definition of the locations where images should be acquired. This might be straight-forward, if samples are arranged in a regular geometrical manner, e.g., on multiwell slides or spotted gene probe arrays. For certain specific, predominantly medical applications, there are whole slide imaging systems available, and "sample localization" strategies in this context have recently been discussed (6). In such cases, the locations of the samples to be imaged are generally known in advance and may be represented as a list of coordinates.

However, some microscopic preparations feature an undefined arrangement of samples on slides. The analysis of aquatic bacteria is a typical example: cells are concentrated on polycarbonate membrane filters (7) and fluorescently stained, e.g., by 4',6-diamidino-2-phenylindole (DAPI) (8) and fluorescence in situ hybridization (9,10). Pieces of membrane filters are then placed on glass slides and evaluated by microscopy. Automated imaging on such preparations renders the creation of a list of coordinates more difficult, as the numbers and the locations of the samples on the microscopic slides is unique and not known in advance (Fig. 1A). Moreover, membrane pieces may exhibit different sizes and geometries, which have to be taken into account when coordinates are defined to ensure systematic uniform random sampling (11). For high-throughput imaging of such undefined samples, it is necessary to have a flexible tool that allows for rapid sample localization on individual slides.

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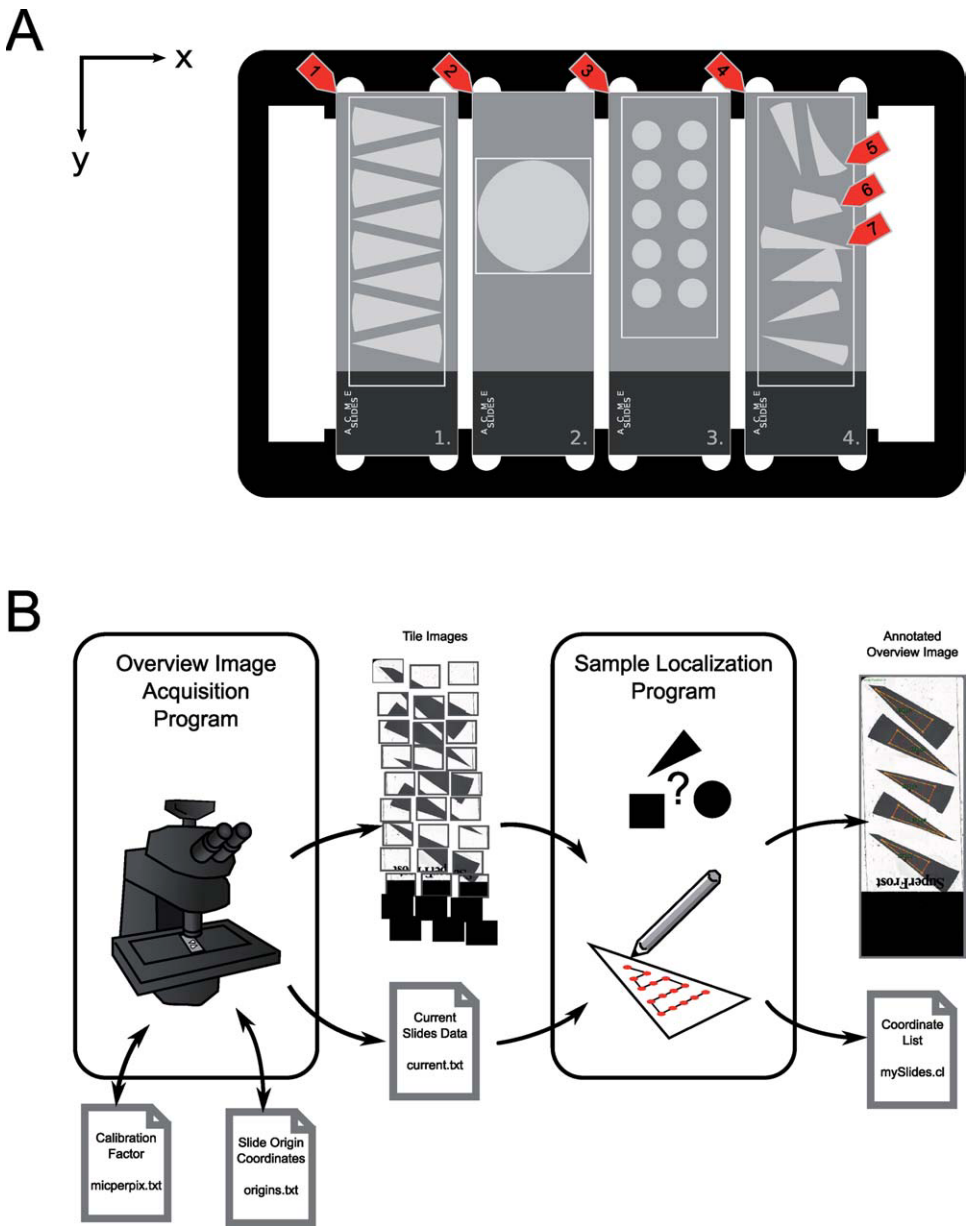

Figure 1. **A:** Schematic depiction of a microscope stage holding four glass slides carrying different types of samples: triangular circle segments of membrane filters, an entire membrane filter, a multiwell slide, and realistically cut membrane filter pieces. **B:** Description of the workflow: two computer programs, symbolized by the boxes with rounded corners are used. The first program images whole slides, resulting in tile images that are passed to the second program, which assembles the image and detects samples. The user is able to modify detected samples and to name them. A file containing a list of coordinates and sample names is produced to serve as input for the subsequent high-throughput imaging routine. Additionally, an annotated overview image is stored for documentation purposes. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

Our solution to this consists of two computer programs and works as follows: in a first step, whole-slide overview images (SOIs) are generated, using a 1× objective and image stitching (or a webcam). In a second step, the overview images are subjected to image analysis for the detection of samples (e.g., membrane filter pieces). Detected samples are analyzed for their geometrical shape (circles, triangle, and rectangles) and a user defined number of coordinates are defined within the shape. Detected shapes may be removed or adjusted by a graphical user interface, and new shapes maybe defined, to allow for greatest flexibility. Our solution was implemented on several Zeiss and a Nikon microscope and the programs are freely available (http://www.technobiology.ch).

Material and Methods

General Strategy

To render our sample localization approach as generic as possible and compatible to microscope systems of different manufacturers, the workflow (Fig. 1B) involves two independent

software programs. The first one, subsequently referred to as "overview image acquisition" involves direct interaction with the microscope stage and camera and is, therefore, hardware and manufacture dependent. Its purpose is to acquire and save tile-images of the glass slides on the microscope stage. The second program, termed "sample localization", is responsible of assembling the tile-images to whole-slides overview images. Subsequently, it has to detect the samples on the slides by image analysis and to allow the user to modify the number, positions, and sizes of the samples on the slides. Importantly, the user has to give a name for each sample. Finally, the program produces two output files. First, a whole-stage overview image depicting all slides and annotation of all samples, their names, and the points where images are to be taken for documentation purposes. Second, a file (plain-text) containing all coordinates of all samples is produced. This file, termed "coordinate list file," is subsequently used as input for the image-acquisition routine.

Microscope Settings

The software was developed on an epifluorescence microscope (AxioImager.Z2m, Zeiss, Germany), equipped with both, epi- and transmission illumination light-emitting diode (LED) devices, a digital camera (AxioCam MRm, 1388 × 1040 px, Zeiss), and a motorized stage capable of holding eight glass slides (225 × 85, PILine, PI, Germany). Overview tile images were acquired using a 1× objective (EC Plan-Neofluar, NA: 0.025, Zeiss). The microscope was controlled by the Software AxioVision 4.8 (Zeiss) and automated using the Visual Basic for Applications (VBA) module within AxioVision.

Program Description: Overview Image Acquisition

The acquisition of tile images was realized by a VBA program within AxioVision. Before the first usage, the camera has to be perfectly aligned to the stage, the calibration factor (μm px^{-1}) for the 1× objective has to be assessed, and the origin coordinates of each slide has to be determined (Fig. 1A, 1–4). The program features a calibration routine, a description is provided in the Supporting Information (Part A; Supporting Information Fig. 1). The program allows for a custom selection of slide position on the stage. After tile image acquisition (default: 3 × 11 tile images per slide), tile images are stored (eight bit grayscale, JPEG file format) and a plain-text file, containing information about calibration, number of slides, origins, and tile images is created. Detailed information on the file format specifications are given in the Supporting Information (Part B).

Program Description: Sample Localization

The sample localization program was created in Microsoft Visual Basic .NET 2005 (VB.NET). It uses the information text file and the tile images to assemble whole slides overview images. Tile-images are resized by a user defined factor (default: 12, resulting in a pixel size of 73 μm) to fit on a standard computer screen and assembled to obtain a SOI. The SOI is finally analyzed to detect samples using a VB.NET image-processing library (Zeder, unpublished data). In a first step, the mean gray value (MGV) of the SOI is analyzed to determine whether the slide is a multiwell slide (MGV $\leq$ 100). Otherwise, the image is inverted. The SOI is three times filtered by a lowpass filter (kernel size: seven) and segmented by a fixed threshold of 128. Binary regions (BR) are traced by flood filling (12) and the following characteristics are measured: area, perimeter, circularity, center of gravity, and the list of contour points. If the area of a BR is in the interval of 0.01–0.4 times the SOI area, a sequential test of possible shape geometries is performed (circle, triangle, rectangle, or none). The algorithm for shape recognition on an arbitrary contour is described in detail in the Supporting Information (Part C; Supporting Information Fig. 2). Recognized shapes are slightly resized and displayed as an overlay on the SOI. The user is able to move, resize, and delete existing shapes as well as to add new shapes. A defined number of coordinates for later imaging is placed in a regular geometrical way within the shapes. The number maybe adjusted for each shape individually. Each shape, depicting an individual sample, has to be named by the user (this sample name information can be used by later screening and data evaluation routines). Finally, a coordinate list file and a composite image of all SOI with annotated shapes and names are stored (Supporting Information Fig. 3). The graphical user interface with a short description of its functionality is depicted in the Supporting Information Figure 4.

Alternative Modes

We propose three alternatives to using a 1× objective to acquire tile-images for the generation of overview images. Blank slide images: the sample localization program may be used without an overview image. A mode is implemented that allows to draw sample shapes onto a user defined rectangular grid on an empty image representing a glass slides (76 mm × 26 mm).

Computer generated templates: artificial overview images of slides may be created, using a computer graphic program (e.g., Inkscape), to serve as a template. Samples have to be drawn as black regions on white background. This approach was used in this study to create a set of different sample geometries for testing the sample shape recognition algorithm (Supporting Information Fig. 1).

Webcam: overview images may be acquired by a webcam instead of the microscope camera. A commercially available webcam (Logitech C905, 1600 px × 1200 px) was attached to a Nikon epifluorescence microscope (Nikon Eclipse 90i). The webcam was controlled by a VB.Net program and the microscope by the MetaMorph (MetaMorph 7.5.6, Molecular Devices) software. The webcam was able to capture an entire glass slide at once. The slide images were then trimmed to the region of the glass slide, resulting in images of 249 px × 738 px (103 μm px^{-1}). These could then be processed with the sample localization program. The programs and detailed descriptions are available at (http://www.mpi-bremen.de/automated_microscopy), for questions contact A. Ellrott (aellrott@mpi-bremen.de).

Performance Test

The duration for image acquisition was measured for both the Zeiss (1× objective) and the Nikon (webcam) microscope. The duration of the image analysis to detect samples was also assessed. The spatial precision of the coordinate list was elucidated using a custom made calibration slide (containing a black stained cover slip: 24 mm × 60 mm). A rectangular shape was drawn targeting the four edges of the black cover slip. The output coordinates of the sample localization program were subsequently checked using a 10× objective and deviations were measured. The success rate of the automated sample detection algorithm was tested on 24 representative slides from recent biological experiments of different researchers and on eight computer generated SOIs containing various artificial shapes, including triangles, circle segments, circles, rectangles, and none-shapes.

Results and Discussion

This work presents an efficient method for generating lists of coordinates for high-throughput imaging of nonregularly allocated samples on microscopic slides (Fig. 1A). The method is based on two separated computer programs (Fig. 1B), one responsible to acquire tile images of glass slides by microscopy, either using a low magnification objective (1×) or a webcam. The second program subsequently assembles the tile images and automatically detects samples (if they are present as circular, triangular, or rectangular dark areas on a light background). A graphical user interface (Supporting Information Fig. 4) allows for adding, moving, and removing shapes to offer greatest flexibility.

The performance of the method is as follows. A single slide is imaged in 40 s when tile images are acquired (1× objective, 3 × 11 tiles), or in about 15 s when a webcam is deployed. Image assembly and sample detection on a single slide is done in ∼ 4 s on a state-of-the-art personal computer. The total time for the user to acquire overview images and to annotate samples on a standard microscopic stage carrying eight slides, with eight samples each, takes, thus, about 10 min.

The spatial precision of drawing coordinates was measured to be 56 ± 58 μm in x-direction and 135 ± 62 μm in y-direction on the Zeiss microscope (using the 1× objective, n = 8). The Nikon microscope equipped with the webcam featured a precision of 243 ± 107 μm in x-direction and 98 ± 58 μm in y-direction, respectively (n = 29). It has to be mentioned, that overview images are resized by a user defined factor (default: 12) to fit on a computer screen. The size of a pixel on the overview image, where the user is able to click and define shapes, represents 73 μm (103 μm on the Nikon system). In this context, and considering the fact that glass slides are fixed only with a clip-mechanism in the slide holder, the precision is satisfactory. To ensure all coordinates to be within the sample area, the automatically placed shapes are slightly downsized—a safety distance of ∼ 0.5 mm to the border of the sample turned out to be optimal.

Regarding the efficiency of the automated shape recognition algorithm, 113 sample shapes out of a total of 117 samples on 24 realistic SOIs (Supporting Information Fig. 3A–D) were correctly recognized. This corresponds to only 3% false negatives, and there were no false positives. Most of the shapes (n = 106) were circle segments, as preparations usually emerge from round polycarbonate membrane filters. A multiwell slide and a single entire membrane filter are shown in Supporting Information Fig. 3C (Slides 6 and 7). There are several reasons for detection failures: on one hand, the program automatically discards shapes that touch the upper or lower edge of the glass slides (Supporting Information Fig. 3C, Slide 5). This is to prevent damage to the objective due to imaging to close to the metal slide holder. On the other hand, shapes may not be recognized if their shape deviates too much from the ideal. This may be due to irregular membrane filter cutting in the sample preparation step (Fig. 1A, 5 and 6; Supporting Information Fig. 3C, Slide 3) or if membrane filter pieces are placed in close proximity so that they touch each other (Fig. 1A, 7; Supporting Information Fig. 3A, Slide 8), thereby being recognized as one single object in the segmentation process. The algorithm is designed to not recognize sample geometries that differ too much from circular, rectangular, and triangular, or that are not within the desired size range. The range of detected and not detected shapes is demonstrated on computer generated shapes (Supporting Information Fig. 3D).

In summary, we developed a highly flexible method to create lists of coordinates for high-throughput microscopic imaging. We demonstrated the applicability on microscopes of different manufacturers. Whole SOIs are a central element in our strategy. We have shown different ways to generate overview images, e.g., using a low magnification objective, an external webcam, or *in silico*, by means of blank images or computer generated templates. Automated sample detection by image analysis allows the creation of lists of coordinates for imaging microscopic slides with completely irregularly arranged samples of different shapes in a short time (eight slides containing eight samples each: 10 min). The user is able to quickly adjust the detection, to name samples and to determine the amount of coordinates within individual samples. Coordinates are placed in a systematic uniform random manner within the detected shape to ensure steady sampling. The need of such lists of coordinates is a indispensable prerequisite of any microscopy based screening application. Here, we offer an approved and comfortable strategy and freely available software.

Acknowledgments

The authors thank Jakob Pernthaler and Eugen Loher for valuable comments and technical support, Tamara Deubelbeiss for helping on vector graphics design, and also the people behind the open source projects Inkscape, Zotero, and OpenOffice.org.

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