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REVIEW

Computer Image Analysis of Microbial Colonies

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Abstract—This review provides a concise description of the basic principles and options of computer-based image analysis for counting and characterization of microbial colonies. Properties of several modern commercially available computer/automated colony counters are described. A number of original works are used to exemplify the new methodological fields made possible by computer image analysis, such as identification of microorganisms according to the external appearance of their colonies, as well as characterization of physiological and biochemical properties and genomics of microbial cultures based on colony growth dynamics. The conclusion is drawn that computer analysis of microbial colony images is a promising way to achieve a qualitatively new level of performance in many conventional techniques and develop novel procedures and analytical systems.

Key words: computer-based image analysis, microbial colonies, computer-aided colony counter, identification of microorganisms, microbial physiology, microbial biochemistry, microbial genomics.

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In 1877, Julius Richard Petri, working in Robert Koch's laboratory, invented a unique technique of microbial cell cultivation in cylindrical plates (dishes) on the surface or in the thickness of gellike nutrient medium to obtain colonies detectable with the naked eye. Many secrets of the microbial world were uncovered with the aid of this seemingly plain technique. The methodology of investigation of the characteristics of microbial colonies is not obsolete and is still widely used in basic research. For example, since the early 1990s, organization of microorganisms within colonies has been studied as a community with various types of intercellular communication [see reviews 1–5]. Characterization of microbial development on petri dishes forms the basis for numerous applied techniques used in modern laboratories of biotechnological, sanitary, epidemiological, and medical specialization, including cases in which it is prescribed by various regulatory documents. However, microbiological analyses on petri dishes are known to be labor- and time-consuming and biased. Procedure of analysis on petri dishes is being improved in several ways [6]. Computer-based image analysis is among them.

This review focuses on a brief description of the major feasible ways automatic image analysis may be presently used as a new instrument for perfection of the techniques associated with cell colony count and characterization.

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PRICIPLES AND CAPABILITIES OF COMPUTER-BASED IMAGE ANALYSIS

Varied characteristics of an object are presented through its image; that is, the color and its saturation and spatial distribution; size, shape, and number of individual parts and their relative position; and, in particular cases, light emission and its brightness. For this information to be useful, these parameters need to be digitized. However, measuring them manually is labor-consuming to such an extent that it makes large-scale tests practically impossible. First of all, as a basis for computer image analysis, a digital picture is transformed into a "mathematical image" that may be further processed quantitatively by computer. Thus, quantitative characterization of an object by its image is significantly accelerated for practical application. Computer analysis makes it possible to use selected components of an image for measurements and calculation, as well as to "edit" the images highlighting the fields of particular optical properties, morphology, etc. The principles of computer image analysis are described in detail in works [7–9].

There is no universal software for image analysis. It would have to be extremely large and thus unmanageable for practical use. This kind of software is typically developed for particular equipment that deals with image acquisition and treatment, such as densitometers for scanning electrophoresis gels (for example, a GS-800 Calibrated Densitometer, Bio-Rad, United Kingdom), optical microscopes (for example, Nikon ACT-1 for L-1, Nikon, Netherlands), or computer colony counters (see below). In addition, there are commercial software packages (for example, Image-

Pro Plus, Media Cybernetics, Inc., United States; MCID™ Core, InterFocus Imaging Ltd., United Kingdom; and Lucida 5.0, Kinetic Imaging, Liverpool, United Kingdom), as well as those available on the net (Image J, National Institute of Health, United States, http://rsb.info.nih.gov/ij/), which make it possible to perform the most frequently required types of image analysis operations. Optical density (or, on the contrary, light emission) measurement in the specified image regions, including measurement in constituent colors (red, blue, and green), is among such types of analysis. The parameter is usually evaluated in gray color gradation units from 0 (black) to 256 (white). Morphometric measurements of linear dimensions and areas are possible, including measurements of objects of complex configuration. Finally, these software packages can count the objects with specified parameter values and perform statistical calculations.

COLONY COUNTING: COMPUTER COUNTER

The first apparatus for automated colony counting was described in 1957. The principle was based on scanning the petri dish with a CRT beam. Upon passing through the dish, the light flow was registered by a photomultiplier [10]. The equipment did not earn wide use. In the 1970s, the first apparatuses employing computer analysis of digital images were designed for automation of microbial colony counting. The principal parts of an automated colony counter are a video camera to capture digital images of the samples (petri dishes) and a computer equipped with appropriate software [6]. Several computer colony counters of this kind are presently available on the market. A brief description of the equipment is given in the table.

First of all, computer colony counters create digital color images of the samples on petri dishes and store them in a computer. The picture may be captured under various modes of illumination—at least, image recording in transient and incident light is always provided. The image quality depends upon the resolving power of the equipment. Usually, it is presented as two parameters, the number of light-sensitive elements of the digital camera (matrix size expressed in millions of pixels) and/or size of the smallest colony that may be detected in the picture. The former indicator is an objective, although incomplete, characteristic, as the apparatus may be equipped with a system of either optical or computer scaling of the registered image. The latter depends on the way the smallest detectable colony size was estimated. Importantly, in practice, the smallest detectable colony size is determined not only by the characteristics of the image registration system, but also by the optical properties of colonies and medium and by illumination conditions. An apparatus with a camera matrix of 0.3 megapixels and the ability to detect colonies of over 0.3 mm appears to be suitable for most practical applications. In some colony counters, software resources allow to edit images the same way it is performed with dedicated software such as Photoshop (Adobe Systems Inc., United States) or ACDSee (ACD Systems Ltd., United States).

All colony counters are equipped with software to automatically count the number of colonies on a digital image of the petri dish. However, strictly speaking, they enumerate the number of elements with a definite set of properties, rather than the number of colonies. Therefore, the choice of criteria to distinguish the elements for counting is vital to obtain adequate results. Most frequently, the optical density and/or the size of elements (colonies) are used as such criteria for petri dish examination in transmitted light. These criteria may also include the color and/or shape of colonies or their various combinations, including those with size and optical density. Computer setting (a kind of "education for the software") is usually performed with a single representative image out of a series of typical (in terms of the medium characteristics, colony shape, etc.) samples. In many apparatuses, some typical criteria, for example, colony optical density and size, are introduced initially (by default). These are automatic settings. However, it is quite evident that, without "tuning" of the equipment in each particular case, there is a risk of inadequate data acquisition. The major problem the user encounters while in the automatic mode is the presence of colony conglomerates. Although many counters provide an option to discriminate between individual colonies within a conglomerate, this is actually realized only in case of rather small conglomerates. In any case, automatic counting is performed with a certain error, the value of which depends on the number of merged colonies.

Many counters possess a function of manual (visual) colony counting on digital images by marking the colonies with virtual markers and record the results of counting in the form of tables. It turned out that visual counting using digital images is faster and more convenient than the conventional mode. With the equipment, an experienced operator may count colonies at a speed of two to three colonies per second. Visual counting largely resolves the conglomerates issue and is more accurate than automatic counting. The principles and capacities of computer colony counting in the automatic and visual detection modes are described in detail in [11].

When interpreting the results of colony count on petri dishes, it should be remembered that the count rate does not necessarily correlate with the number of viable cells in microbial samples. First of all, the correlation is true only for cell cultures that naturally grow as individual cells not forming any aggregates. However, even in such cultures, partial aggregation may be induced by certain laboratory manipulations—for instance, during the sample preparation. In any case, to reveal the aggregates, the samples are to be analyzed

Main characteristics of computer colony counters of various manufacturers

Model, company	Image registration		Colony count				Data storage		Other options
	Illumination	Matrix*, Mpix	Min. size, mm	A**	V***	Settings	Original images	Counting results	
SCAN 500 Colony Counter (Topac, United States)	Transmitted and incident light, dark field, and light field	0.3	0.1	+	+	Automatic	Without editing	Proprietary format, export to MS Excel	Work with conventional and spiral inoculation, automatic correction of medium defects
Clinx BioCounter 1200 (Clinx Science Instruments, China)	Transmitted and incident light	1.4	0.05	+	+	Automatic, "manual" tuning	Without editing	Proprietary format, export to MS Excel	Measurement of area, perimeter, linear dimensions, optical density, etc.
Color QCount (Sysmatec, Germany)	Transmitted and incident light, dark field with polarization	1.3	0.2	+	+	Automatic, "manual" tuning and setting	Allows editing	Proprietary format, export to Laboratory Information Management System (LIMS) format	Automatic contour analysis and cluster identification
Schütt colonyQuant (Schütt Labortechnik, Germany)	Transmitted and incident light, autofocus	0.3	0.1	+	–	"Manual" setting, also by sectors	Allows editing	Proprietary format, export to MS Excel and Laboratory Information Management System (LIMS) format	Independent counting of colonies differing in color, shape and size, analysis of growth suppression zones
Sorcerer Colony Counter (Perspective Instruments, United Kingdom)	Transmitted and incident light, dark field	0.4 (1.3)	0.15 (0.08)	+	–	Automatic and "manual" setting, also by sectors	Allows editing	Proprietary format, export to Laboratory Information Management System (LIMS) format	Counting in individual sectors of different configuration, computer "filters" to discriminate colonies by shape, size, density, etc.
ProtoCOL SR, HR (Synbiosis, United States)	Transmitted and incident light, dark field	–	0.09 (0.08)	+	+	Automatic and "manual" setting	Without editing	Proprietary format, export to Laboratory Information Management System (LIMS) format	Computer filters to discriminate colonies by shape, size, and color. Measurement of growth inhibition zones
KOMPANKOL-M1 (NABITECH, Russia) [6]	Transmitted and incident light	0.3	0.3	+	+	Automatic and "manual" tuning and setting	Without editing	Proprietary format, export to any MS Office application	Russian interface, independent count of colonies differing by color, independent counting in sectors of differing configurations, measurement of the selected fragment

Notes: * Camera resolving capacity, megapixels.
** Automatic mode of colony counting.
*** Visual mode of colony counting.

by microscopy before inoculation onto petri dishes. Typically, aggregates are broken down by intense mixing, for example, by means of repeated pipetting. In case it does not help, some authors recommend short-time treatment of samples with ultrasound or detergents. However, it is practically impossible to determine whether these factors are not damaging to the entire cell population. The samples containing cells character by differing optimal growth conditions are particularly difficult for evaluation of viable cell content by colony forming ability. These may be cell consortia of different species or populations of a single species containing damaged or so-called viable but nonculturable forms [12].

In the table, some peculiarities and extra options of the computer colony counter software are presented. In particular, a tool to measure the size of objects in the image is worth noting. It extends significantly the area of equipment application for research as well as practical needs. For example, Clinx BioCounter 1200 (Clinx Science Instruments, China), Schütt colonyQuant (Schüt Labortechnik, Germany), and ProtoCOL SR, HR (Synbiosis, United States) software packages provide an option to measure the size of growth inhibition zones for antimicrobial activity testing. The possibility to automatically count yeast cells in the Goryaev chamber by means of digital image processing with the KOMPANKOL-M1 (NABITECH, Russia) software package was demonstrated [11]. Some apparatuses include software for statistical data treatment.

COLONY MORPHOLOGY AND IDENTIFICATION

External appearance of the colonies is one of the essential phenotypic characteristics in microbial identification. The major parameters of a colony used for identification are coloring, texture (mucous, dry, pastelike, loose, dense, or other) shape (convex, flat, conical, or other), surface nature (smooth, wrinkled, rugulose, or other), and edge type (smooth, villous, or other). The differences in colony appearance may be distinctive features for differentiation at the level of various taxonomic groups, including strains of a single species. Importantly, all these characteristics depend upon cultivation conditions—primarily, on medium composition, temperature, and time. Although colony appearance cannot be the sole parameter used for identification, under standardized culturing conditions, it may be used in a number of practical approaches when culture express diagnostics is required. Searching for a culture with certain characteristics by screening, control of the state of isolated cultures, disease diagnostics, sanitary and epidemiological expertise, etc., are situations of this kind. Computer image analysis may be helpful in such cases. Let us concentrate on some examples of successful application of this approach.

A special software based on multifractal analysis [13] was developed for quantitative morphology characterization of the fungus *Metarhizium anisopliae* colonies using 14 parameters. The software provided fast identification and classification of the colonies of individual strains possessing varying activity of steroids biotransformation. Moreover, the software allowed more objective strain selection. Comparison of computer and visual analyses results showed 96% agreement.

Potential for the identification of the *Penicillium* fungi by optical properties of their colonies using computer image analysis was also studied. Identification procedures were developed to identify the fungi of the genus *Penicillium* at the species level [14, 15] and *Penicillium commune* at the level of clones by color and texture [16]. The species-level identification technique involved the combination of cultivation conditions and sample illumination, which allowed for 100% correct determination in the validation tests on three fungal species [14]. In case of *P. commune* identification at the level of clones, cross validation of the method with DNA fingerprinting of 137 isolates demonstrated 93–98% agreement [15].

A system was developed for identification of bacteria of the genus *Listeria* at the species and strain level [17]. The system is based on registration of the optical image of the colonies created by laser beam scattering followed by computer analysis of the sample. An optical image database of 108 strains of six bacterial species of the genus was created which allowed identification of the cultures with 91–100% accuracy.

As major advantages of the identification techniques, the authors of the above studies particularly emphasize its objectivity and the possibility to use them without necessity for "visual training" of the personnel.

COLONY DEVELOPMENT DYNAMICS AND PHYSIOLOGICAL AND BIOCHEMICAL PROPERTIES OF CELLS

Dynamics of colony growth on solid nutrient media provides information on surface growth of microbial populations in their natural habitats. Computer image analysis may be used to automate the registration of development of individual colonies and thus to obtain statistically reliable data. For example, registering dynamics of colony formation for *Listeria monocytogenes* cultures was used to determine the lag phase duration of the culture on solid medium and to compare it with the value for the culture grown in liquid medium [18]. Thus, it was demonstrated that upon exposure to stress factors, the lag phase duration changes to the same extent in the cultures grown on both solid and liquid media.

Registration of colony size dynamics (colony growth) was used to study the effect of 17-alpha and 17-beta estradiol isomers on an estrogen-dependent

Candida albicans strain [19]. Significant difference in colony growth rates in the presence of these two isomers was revealed, suggesting the probable involvement of sterols in the regulation of fungal metabolism, particularly in the process of virulence development.

A system was created for automated quantitative data acquisition on *Saccharomyces cerevisiae* colony growth when inoculated as a matrix [20]. The system, termed Growth Detector, is aimed at functional genomics experiments requiring analysis of large data arrays on colony size measurements. With the system, a relationship was established between the activity of berberine, an antifungal compound, and certain components of gene expression. A new link between the *mek1* gene and the DNA reparation system was found. It is expected that the methodology based on computer image analysis for monitoring of the colony growth dynamics will promote the development of studies connected with soil microbial populations [21].

COLOR REACTIONS IN MEDIUM AND SCREENING

Another example of the task facilitated considerably by automatic computer image analysis is screening of the enzymatic activity of microbial cultures by reactions in the cultivation medium. An example of a successful application of the methodology is a highly efficient analytical system to reveal enzymatic activities by the development of small-size colonies on special filters [22]. The system allows for simultaneous detection of activities in 7000 microcolonies. During validation experiments with esterases, the system was shown to be able to discriminate between the activities of cells of colonies differing by 10–20%. The work performed on the microcolonies of the fungus *Pycnoporus cinnabarinus* [23] should also be mentioned in this connection. The work revealed a correlation between hyphae branching degree and secretion of acid phosphatase and laccase into the medium by means of special software tools based on fractal analysis [24].

To conclude, the methodology of computer image analysis is a promising way to impart a qualitatively new level to many traditional procedures dealing with characterization of microorganisms by their development as colonies on solid media. It is a more advanced level of analysis of the information carried in optical image of a microbial colony. The methodology yields faster and more objective analyses. The technical equipment is commercially available; original methodological approaches were developed. However, it is merely an instrument, as any methodology is, and its successful application depends on the ingenuity of the investigators of the microbial world.

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