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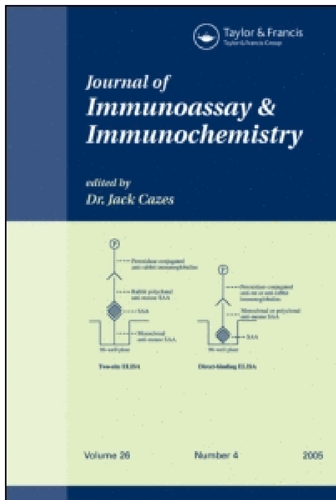

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A FULLY AUTOMATED MICROSCOPE BACTERIAL ENUMERATION SYSTEM FOR STUDIES OF ORAL MICROBIAL ECOLOGY

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A FULLY AUTOMATED MICROSCOPE BACTERIAL ENUMERATION SYSTEM FOR STUDIES OF ORAL MICROBIAL ECOLOGY

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ABSTRACT

A fast and fully automated image analysis technique for the enumeration of fluorescence-labeled bacteria in oral samples

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was developed. This paper describes the system configuration, application strategy, automated operation, and initial validation experiments using fluorescent microspheres, bacterial cultures, in vitro grown biofilms and human dental plaque. Following a series of brief operator-controlled calibration steps, the technique automatically performs all necessary microscope operations (stage translation, focus, sampling and analysis) on slides with up to 48 wells for as many different samples. It quantifies bacteria from differential interference contrast images, images showing cells that had been labeled by immunofluorescence with monoclonal antibodies, or images with cells labeled by a fluorescent DNA stain.

With all evaluated samples, close agreement between the automated system and the assessor's visual counts was observed. This novel automated image grabbing and analysis procedure is applicable to the enumeration of specific taxa in clinical samples by both immunofluorescence and fluorescent in situ hybridization.

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INTRODUCTION

The importance of complex bacterial biofilm communities in the human oral cavity is the subject of intense research.(1) A key parameter to understanding the ecological role of bacteria in the oral environment is the quantification of specific bacterial populations. Most of the current knowledge of the composition of the oral microbiota derives from classical cultural methods.(2 for review) However, estimates from molecular studies show that typically a significant fraction of the sample cannot be cultured.(3,4) In addition, it is widely recognized that culture analysis is time consuming, expensive, laborious and neither a precise nor accurate method.(4) A number of alternative methods for the detection of organisms have been developed (e.g. immunofluorescence (IF), fluorescence in situ hybridization (FISH), flow cytometry) that rely on 'probes' – either antibodies or complimentary DNA – with exquisite specificity for selected bacteria.(5–9) Particular advantages of the IF and the FISH approach include single cell resolution and visual confirmation of object morphology; this implies direct recognition of the key 'identifiers' by the experienced observer. However, the accuracy of direct epifluorescence counting methods also depends on the staining techniques used and the physico-chemical characteristics of the samples. (10–12) Therefore, significant precautionary measures, considering individual investigator bias, must be taken into

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account if accurate and reproducible quantification is to be obtained from direct microscopy.

To limit assessor bias, semi-automated 'direct observation' enumeration methods utilizing combinations of image analysis capabilities and partially automated microscope or confocal microscope systems have been reported by several authors.(11,13–17) Many of these initial systems were only semi-automatic since unwanted detrital material had to be removed by time consuming interactive image editing and thresholding. However, given the complexity of natural oral ecologies with regard to both composition and physical structure, the key enumeration requirement is the identification of individual species, without extensive image editing, and this over a wide range of target organism diversity. It is clear, therefore, that any direct microscopy assay method must be capable of large-scale sampling and of providing a fast, comparable and robust analysis.

The aim of this project was to develop a versatile microbial detection system for oral ecology studies of both in vitro and clinically derived samples. In particular, the new instrument was required to be at least as fast as a human operator; accurate, reliable, specific and very sensitive. In the present paper we describe in sufficient technical detail for reproduction a complete system capable of independent operation which, following a series of brief operator-controlled calibration steps, automatically performs all necessary stage translation, focus, sampling and analysis steps for up to 48 samples. We further describe a series of validation experiments carried out to investigate the system's performance using rather simple samples. In parallel, extensive IF and FISH validation studies with much more complex structured dental plaque samples were carried out and published in two complimentary papers.(18,19)

EXPERIMENTAL

System Configuration and Hardware

An Olympus BH2-BHS microscope (Olympus Optical Co (Europe) GmbH, Hamburg, Germany) equipped for differential interference contrast (DIC) and epifluorescence was used. Identification and enumeration of individual organisms was achieved using an Olympus DPlanApo100UV objective with a numerical aperture of 1.32 in combination with 10× eyepieces and 3.3× or 5.0× photo-oculars. A single narrow band pass filter set (Olympus IB) was used for all fluorescence observations. Imaging was performed with a low light level SIT camera (C2400-08; Hamamatsu Photonics GmbH, Hersching, Germany). As two imaging modes with differing

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illumination requirements were to be used, the microscope was modified by incorporating three pneumatically controlled 2-position actuators (435 00 297 with Isoclair C10 AS 50; ASCO Joucomatic S.A., Rueil, Malmaison, France) as filter changers. By closing actuator A3 (block-transmitted light) and opening A1 (fluorescence illumination) and A2 (removing polarizing filter), fluorescence illumination conditions were obtained. By closing A1 and opening A2 and A3, DIC was obtained. To facilitate the automatic switching by the image processing system (serial communication line), a simple binary interface unit to control the actuators was constructed.

Sample (multi-well microscope slide) translation was achieved using a Marzhauser (Wetzlar, Germany) programmable motorized (x-y) stage fixed to the microscope and fully controlled by the image processing system. For autofocussing, the microscope was equipped with a piezo-electric objective translator (P-20.10 PIFOC and P862-50 controller; Physik Instrumente GmbH & Co., Waldbronn, Germany) interfaced to the image analysis system. The device was capable of 100 μm travel at a resolution of 0.01 μm and when fitted with a feedback encoder exhibited position (translation) hysteresis of less than 0.1 μm over its working range. Autofocussing relied on the well known result that 'thin' objects have maximum contrast at best focus and therefore a standard deviation based image comparitor algorithm goes through a maximum at position of best focus.(20) Following successful preliminary tests (data not shown) demonstrating that optimal focal points could be found, even with only a single object within the image field, this method was adopted for all focussing operations.

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Image Analysis System

A Kontron IBAS image processing system (Wetzlar, Germany) controlled all image processing, analysis, decision, and control processes. The system consisted of a host computer (Kontron 486/66 PC) and a Kontron MIAP2 array processor equipped with a frame grabbing facility capable of digitizing 48 images at 8-bit image. The IBAS system operates within a DOS environment and utilizes a flexible proprietary direct microcode interpreter language (version 2.0) as its image processing and control language.

Automated System Operation

Human Computer Interface

User interaction with the instrument was required only when the microscope slide was first located on the microscope. Alignment points were

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taken by driving the stage and objective translator interactively through the IBAS system using a specifically designed graphic interface to the reference points. By taking a relatively large number of reference points (7 for 24 well and 9 for 48 well slides), simple linear interpolation in x, y, and z was found to yield a very good first estimate of focal position, across the microscope slide 'plane'. This minimized the number of steps required by the 'fine' focus algorithm.

Well Scanning Strategy

The enumeration viewing frame was $\sim 50 \times 50\,\mu m$ and, since average bacteria size was of the order of $1\,\mu m^2$, the edge-effect rule for unbiased object estimation in these type of viewing frame experiments was used.(17,21) If the bacteria can be considered as independent and normally distributed objects on the well surface, then a total bacterial count or count based on the fraction of wells examined is sufficient.(13,16,22) With this type of distribution, Gunderson(21) has shown that the most efficient sampling procedure is to scan a raster pattern of images across the well. A further advantage, for an $n \times n$ raster pattern, is that simple scaling rules allow selection of every mth image in the pattern according to the bacterial density and variability encountered.

Apparatus Duty Cycle

The enumeration cycle was composed of the following distinct actions:

(i) Translate the sample to a new area; reset the objective start position
(ii) Implement the objective translation cycle ($\pm 0.5\,\mu m$ for best focal plane detection)

- Grab single image
- Analyze image statistics for focus criterion
- Repeat above over defined range of translation

(iii) Decide optimal focus level, if focus not found (no objects) translate sample to new area.
(iv) Set objective to optimal focus position.
(v) Grab and average image to improve signal to noise ratio, correct for illumination gradients, etc.
(vi) Analyze image for bacterial counts.
(vii) Repeat above.

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DIC Object Segmentation

Segmentation of individual bacteria within each DIC image used the methods of gray level morphology originally developed by Serra,(23) and subsequently described by several other authors.(20,24) It comprised the following steps: detection of aggregates using Top Hat Transformation, Watershed (or Regional Maxima) to detect individual organisms, intersection of aggregates with the watershed basins, application of decision rules to reject unwanted detritus, and enumeration. (As an option, objects were contoured and displayed). Using this approach, the complete bacterial mask could then be directly superimposed on the equivalent image taken in fluorescence mode.

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Fluorescence Object Segmentation

A separate aim was to develop a segmentation algorithm for the recognition of bacterial objects from the fluorescence images alone. Because of the 'halo' effects associated with fluorescence images, the main problems encountered when enumeration or morphometric analysis is required are the definition of boundaries and the selection of appropriate threshold levels.(25) Previous authors have shown that convolution filters produce second derivative transformations which yield gradient images with step edges and, therefore, provide a remarkably robust way of threshold setting.(20,26,27) Furthermore, by tailoring the extent and magnitude of the matrix elements, it is possible to transform the filters such that they are particularly sensitive to the features and characteristic dimensions of the object bacteria (an optical magnification dependent phenomenon). In this work, the optimal filters were found to be the sparse forms of the Laplacian convolution filter. Following application of these filters, additional thresholding and segmentation steps were included to remove high frequency noise and acceptable object recognition. For use of high intensity monoclonal antibody (MAb) stains or the green fluorescent nucleic acid stain SYTOX® (Molecular Probes, Eugene, Oregon), a calibration set principle was developed in which examination of one or two control wells was used to determine the optimum fluorescence threshold level for the subsequent segmentation process. Overall, the combination of fluorescence threshold and convolution filter produced a fast segmentation procedure, which gave very good accord with visual assessments.

Fluorescent Microspheres

Fluorescent microspheres (Polyscience, Warrington UK) were used as photobleach resistant control test materials for the development of the

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imaging apparatus and the enumeration software. Beads of 1.0 ± 0.02 μm diameter were used with fluorescence spectral characteristics selected to be similar to those that would be encountered with fluorescence labeled bacteria. For imaging experiments, beads were diluted and dispersed to the required density (10^3–10^9 beads per mL) and allowed to air-dry onto the wells of microscope slides.

Pure Cultures and Biofilm Samples

Lactobacillus rhamnosus AC413, *Veillonella parvula* ATCC 17745 and *Streptococcus oralis* 34P (provided by Dr. J. Cisar, NIDR, Bethesda, MD) were grown overnight in Brain Heart Infusion medium. Mixed species biofilm cultures were a consortium of strains: *S. oralis* OMZ 607 (= SK 248), *Streptococcus sobrinus* OMZ 176, *Actinomyces naeslundii* OMZ 745, *Veillonella dispar* OMZ 493 (= ATCC 17748^T), and *Fusobacterium nucleatum* OMZ 596. Biofilms were grown on sintered circular hydroxyapatite discs as described by Guggenheim et al.(28) After 30 min, 16.5 h, 40.5 h and 64.5 h biofilms from three discs each were harvested and assessed by culture(28) and automated image analysis (see below).

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Clinical Samples

Subgingival plaque was obtained using paper-points(29) from a deep periodontal pocket of an adult patient with severe progressive periodontitis. Interdental supragingival plaque was obtained from 11 dental hygienists and 10 subjects with severe gingivitis as described.(30)

Preparation and Staining of Microscope Slides

The bacterial sample was suspended in a solution of 0.9% NaCl/ 0.00025% hexadecyltrimethylammonium bromide, typically to a concentration of 10^7–10^8 bacteria per mL. Of this suspension, 7.5 μL or 10 μL were dropped onto each well of microscope glass slides (Cel-Line Associates Inc, Newfield, New Jersey). Slides had 15, 18, 24, or 48 wells of 4 mm diameter, but principally slides with a maximum size of 75 × 50 mm and a freely definable well format are acceptable. The slides were allowed to air dry before fixing with methanol (2 min). In preliminary experiments (data not shown), the surface features of a series of microscope slides from various manufacturers were measured using a non-contact laser profilometer (Rodenstock Laser Stylus RM600-LS10, Mahr, Wilton Keynes, U.K.).

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The flatness of the preferred slides (Cel-Line) varied by less than 10 µm edge to edge across its length and, hence, was acceptable for the desired application of an automated microscope. Individual wells were stained for IF as described(29) using MAbs 116BF1.2 to *Bacteroides forsythus*,(31) 212WR2 to *Campylobacter rectus*,(9) 391FN1 to *F. nucleatum* serovar 12,(30) 61BG1.3 to *P. gingivalis*,(32) 326PM2 to *P. micros*,(33) 396AN1, and 397AN1.1 to *A. naeslundii*,(34) 332LR1.1 to *L. rhamnosus* (Thurnheer et al., unpublished), 348VP1 to *V. parvula* ATCC 17745,(18) R26 (supplied by Dr. J. Cisar, NIDR, Bethesda, MD) to *S. oralis* 34P, and 493SO1 to *S. oralis* OMZ 607 (Gmür et al., unpublished). To enumerate all bacterial cells, 10 µL of SYTOX® was added to each well at a 1:330 dilution in 0.9% NaCl and incubated for 15 min at 37°C. Samples were then washed once with 0.9% NaCl solution and once with distilled water. After drying, slides were mounted as for IF staining.

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RESULTS

Figure 1 shows a schematic of the instrument. Two particularly notable features are a piezo-electric objective holder for highly accurate autofocussing and three 2-position actuators for the computer-controlled pneumatic switch from DIC to IF and vice versa. Figure 2 illustrates step-by-step how the original fluorescence image of *B. forsythus* in subgingival

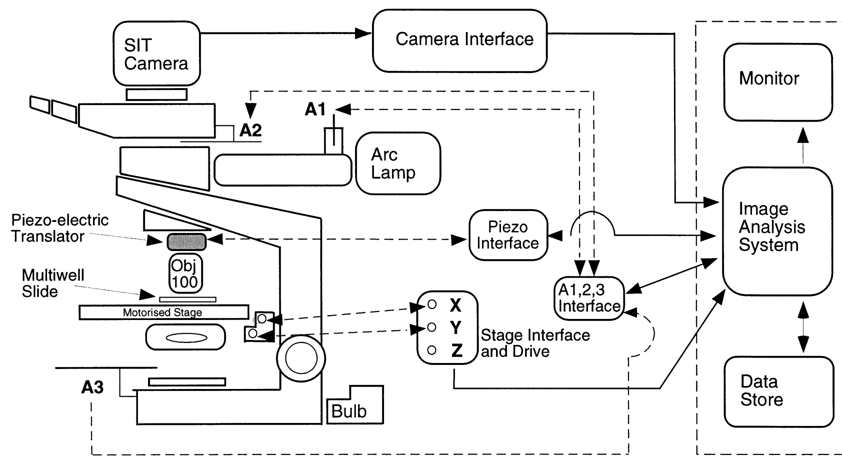

Figure 1. Schematic representation of the components of the automated image grabbing and analysis system.

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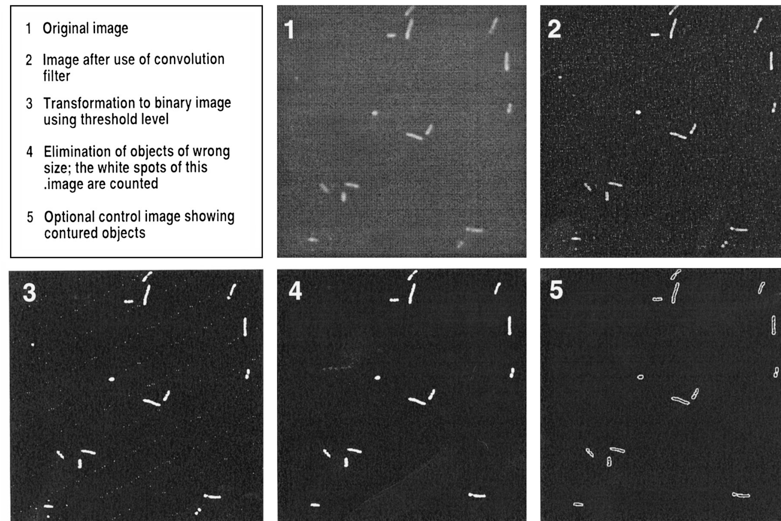

Figure 2. Step-by-step illustration of the fluorescent object segmentation procedure. The image analysis was performed on a subgingival plaque sample stained for *B. forsythus* by IF.

plaque is transformed for bacterial counting. Overall, the combination of fluorescence threshold and convolution filter produced a fast segmentation procedure, which gave very good accord with visual assessments (see below). A routine operation assessing 64 fields in a given well (256 field meander of which every 4th field is evaluated) takes approximately 1100 seconds, that is 15 sec to translate to and get focus of a field, and 2 sec for image analysis and data storage.

Evaluation of Counting Algorithms Using Fluorescent Beads

The primary task of the automated system is efficient bacterial enumeration and, hence, overall system duty cycle and sampling rate are key criteria in defining the utility of the apparatus. Therefore, the most time efficient algorithms were selected for testing, i.e. those for gradient filter (2 sec per image) and gray level morphology (6 sec per image). In addition we wanted to compare the accuracy of the automated system with human experts. Fluorescent beads at a nominal density 1×10^9 per mL were dispersed into wells of microscope slides and enumerated by the automated system against a set of 5 expert assessors. Expert assessments were made on

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digital printouts of the test fields with an effective viewing magnification of at least 2000 times. The comparison of the two algorithms showed that the gradient filter method exhibited better agreement to assessor data than the fast gray morphology (Table 1). Sample data of the assessor to system comparison are shown in Figure 3. Results indicated that the level of agreement between automated enumeration and the expert readings was satisfactory. Agreement was better when the beads were counted at increased optical magnification (Table 1) because of the improved readability of the printouts evaluated by the assessors.

In tests using a wide diversity of test bead patterns and densities, each test algorithm was consistently noted to produce enumeration results with no inherent variability. To investigate the sensitivity and congruency of the instrument, a suspension of latex spheres (nominally 1×10^9 per mL) was serially diluted, down to a nominal value of 10^3 per mL and triplicate volumes of 3 µl were placed onto different wells. These samples were analyzed using a master, 16×16, raster pattern (randomly selected within the limits that would allow the pattern to fit within the sample well) of which, depending on the expected bead density, all fields, or every 4th, 8th, 16th, or 32th field were measured (Table 2). The data from the subpatterns were used to define sampling rules for making efficient estimates of object density.

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Table 1. Summary of the Overall Level Agreement of the Gradient Filter and Fast Gray Morphology Algorithms Compared to Expert Assessors at Two Optical Magnifications (100× Objective)

		Average Deviation of Algorithm from Assessor[a]	
Viewing Mode	Eyepiece Magnification	Gradient Filter	Fast Gray Morphology
DIC	3.3×	1.35	1.84
Fluorescence	3.3×	1.85	2.36
DIC	5×	1.10	2.67
Fluorescence	5×	1.57	2.78

[a]For each image the median in the expert count and the range of values produced by the 4 experts in most agreement was defined. Then the deviations of the automated system (a) from the truncated median expert value (m) with respect to the truncated range (r), calculated from: deviation= (a−m)/r. Hence, these deviations give a measure of the difference between algorithms and 'typical' expert as a function of inter-expert variation. The average deviation can then be defined as the square root of the mean of the summed squares of the individual deviations. A value of around 1.0 indicates that the algorithm could be considered equivalent to another expert. Hence, deviation values near 1 or 2 are seen as satisfactory.

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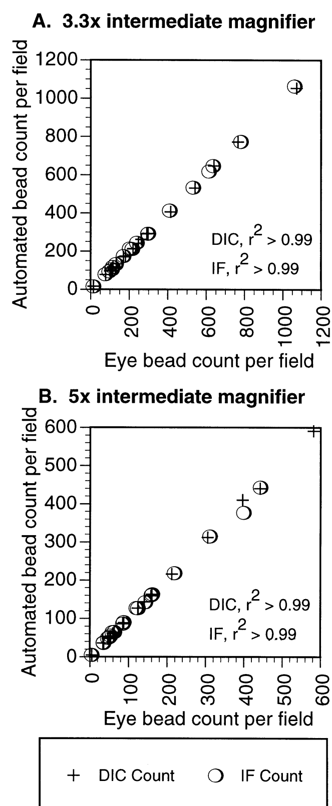

Figure 3. Correlation between automated system and five expert assessors in quantifying fluorescent microspheres. Both DIC and fluorescence images were assessed. Assessments were made of two optical magnifications using (a) a 5× and (b) a 3.3× intermediate magnifier.

Evaluation of Instrument with Clonal Bacterial Cultures and Multi-species Biofilms

We first used a pure culture of small cocci (*S. oralis* 34P) and selected randomly ten image fields – imaged in both DIC and fluorescence – to compare automated enumeration with visual counting. As with beads, the level of agreement between the assessor and the automated system was quite satisfactory (Fig. 4). In this example, the automated system measured a total of 241 bacteria with 93% being fluorescent, whereas the totals were 242 bacteria and 91% fluorescent for the human assessment. Similar experiments with cultures of *V. parvula* and *L. rhamnosus* confirmed these results.

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Table 2. Comparative Enumeration of Fluorescent Beads as a Function of Bead Concentration and Area Mapped

Nominal Bead Density per mL	Number of Viewing Fields Forming the Area Mapped				
	8	16	32	64	256
10^9	4001[a]	9155	21565		
	4654	9713	19998		
	4145	10043	22064		
	4256 ± 343[b]	9768 ± 643	21209 ± 1078		
10^8		643	1819		
		774	1992		
		851	2104		
		756 ± 105	1972 ± 144		
10^7		36	166		
		75	144		
		86	183		
		66 ± 26	164 ± 20		
10^6		14	26		
		5	17		
		6	12		
		8 ± 5	18 ± 7		
10^5			2	4	
			2	1	
			0	5	
			1 ± 1	3 ± 2	
10^4					0
					1
					1
					1 ± 1

[a] Beads counted in area mapped.
[b] Mean number of beads in area mapped ± 1 standard deviation.

Next, in vitro grown multi-species biofilms were assessed in triplicates for *S. oralis*, *A. naeslundii*, and the total number of bacteria. However, in this experiment automated counting was compared to bacterial enumeration by cultivation. In spite of the more complex sample composition resulting in aggregate formation and the entirely different counting principles, total CFU and total optical bacterial counts (SYTOX®-labeled cells) showed a high level of agreement (Fig. 5). The respective numbers detected for *S. oralis* agreed similarly well. With *A. naeslundii*, characterized by strong self-aggregation, CFU values were expectedly smaller than those determined

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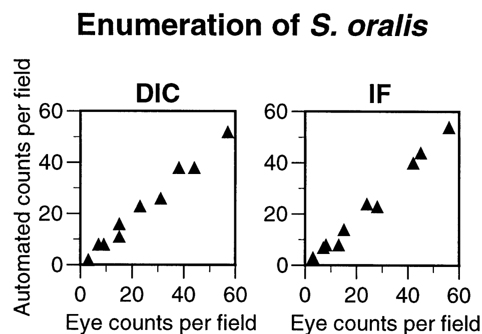

Figure 4. Comparison of determinations from a single assessor and the automated system for culture-derived MAb-labeled *S. oralis* cells. Measurements were made using both DIC (r^2= 0.98, gradient= 0.91) and IF (r^2= 0.99, gradient= 0.97). The data derived from 10 randomlfy selected image fields.

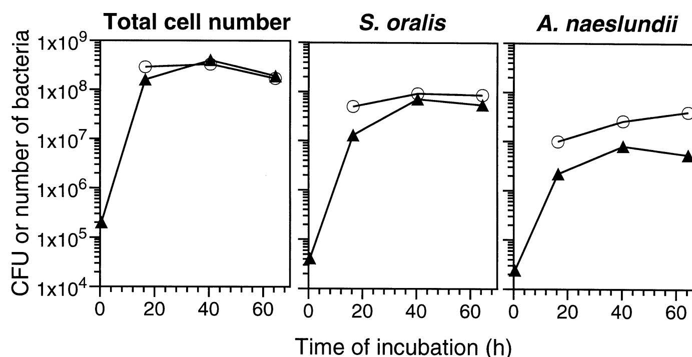

Figure 5. Comparison of determinations from culture and the automated system for biofilm samples collected at various time points. Culture data are presented as CFU (▲), whereas image analysis data represent single cell counts (○). Samples collected at 30 min were assessed only by culture.

by automated IF (Fig. 5). Overall, the data indicated that the automated system was capable of reliable enumeration of single species growing within mixed communities.

Application of the Automated System to Clinical Samples

The ability of the automated system to enumerate specific bacteria in plaque samples was tested using subgingival plaque from a subject

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Table 3. Comparative Enumeration of Selected Taxa from a Subgingival Plaque by Automated and Conventional Visual IF

Bacterial Species	Labeling Reagent	Counts ($\times 10^3$ per Well) Automated (A)	Visual (V)	Ratio A/V
All bacteria	SYTOX®	335.0 ± 47[a]	–[b]	–
P. gingivalis	61BG1.3	61.4 ± 6.5	42.6	1.4
B. forsythus	116BF1.2	78.2 ± 10.2	56.1	1.4
C. rectus	212WR2	7.7 ± 1.3	4.9	1.6
P. micros	326PM2	8.6 ± 2.7	4.6	1.9
A. naeslundii	396AN1	8.3 ± 1.9	4.3	1.9

[a]One standard deviation.
[b]The combination of high bacterial density and relatively rapid bleaching did not allow a visual enumeration.

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with severe recurrent periodontitis (Table 3). Using the automated system, 8×8 raster patterns were defined in 3 randomly selected start positions within each well and in which every second field was enumerated. Visual enumeration was carried out as previously described for plaque samples. Results showed excellent correspondence between the cell numbers determined by the two methods. The scores from the automated system were consistently slightly higher than those determined visually by the assessor. Repetitive automated measurements in two wells of the total cell number of SYTOX® labeled cells yielded triplicate-values of 4561/3178/3367 and 4202/4425/4146, or a mean value of 3980 ± 571 (SD). The system's performance was further tested with interdental supragingival plaque from 11 dental hygienists and 10 subjects with severe gingivitis (Fig. 6). The plots demonstrate good correspondence between the two techniques with correlation coefficients ranging between 0.965 and 0.989 ($p < 0.0001$). Conflicting results were only noted at critically low densities of MAb-labeled cells (below approximately 75 cells per well) due to the inevitable presence of a small number of fluorescent objects that were false counted by automated image analysis.

DISCUSSION

Combinatorial image analysis and microscope systems were first reported in the mid 1980's in the context of plankton enumeration.(13,16) Since then approaches to the enumeration and analysis of both prokaryotic

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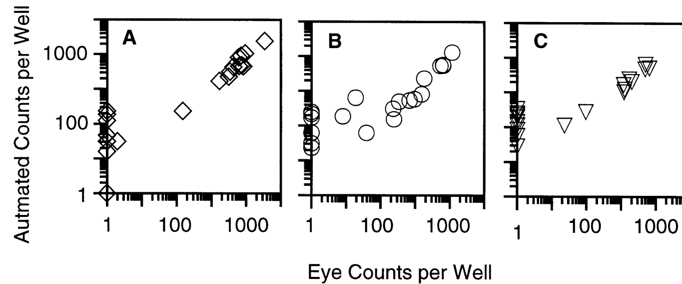

Figure 6. Enumeration of *B. forsythus*, *C. rectus*, and *F. nucleatum* serovar 12 in 21 interdental plaque samples. Results generated by automated analysis are compared to data from visual examinations. To enable log-transformation, values of 0 were replaced by values of 1.

and eukaryotic cells have become ever more sophisticated.(17,35) This sophistication has broadly paralleled the increasing computational ability associated with image analysis systems. However, even though landmark improvements have been made, earlier instrumental systems all required significant input from expert practitioners, such as interactive image editing, manual focus, and stage translations. The key impact of previous systems, therefore, would seem not to result in saved time/effort by the operator, especially if only enumeration data was required, but in more precise and more abundant object specific information, such as morphological data.

The automated system described in this work enables the skilled assessor to otherwise utilize time previously spent on the tedium of enumerating cells in multi-well microscope slides. This includes all stage translation activities, including using variable decision rules to allow effective sampling procedures, all autofocus and enumeration tasks together with collating data for subsequent use. By utilizing a fast overview screening of the entire microscope slide, it is possible to select the enumeration and sampling algorithms either to avoid particular problematic areas or to scan particular interesting areas. Efficient autofocussing algorithms yield a high duty cycle for the system. Efficient enumeration algorithms, based on gradient filtering and training set data for fluorescence imaging, and gray morphology for DIC viewing, yield enumeration results at rates comparable to expert assessors in 2D. Further key advantages of any automated system are the objective and systematic nature of the enumeration determination. These factors imply extremely reproducible interpretations of objects and object patterns, factors, which are known to vary from assessor to assessor and even within an assessment throughout the course of a lengthy experiment. At least two other groups(36,37) have developed simultaneously

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comparable automated image analysis systems for microbial enumeration that are based on microscopes with motorized and software-controlled stages and focus. While Grivet et al.(36) worked with metal alloy disks that had been immersed in cultures of two oral bacterial species, Jansen et al.(37) employed simultaneously up to eight 6-well slides with square-shaped wells that had been coated with fecal suspensions. In comparison to these conceptually similar systems, we use multi-well glass slides with wells of much smaller surface area, saving both precious sample and detection reagents. Furthermore, we work with a piezo-electric objective holder for autofocussing of very high precision and employ a different software solution.

The limits of this automated enumeration system are broadly similar to an expert assessor. Principally, successful operation of the system is governed by the quality of the primary image arriving at the detector. MAbs were selected based on labeling efficiency, level of continuous cell wall labeling of each cell and absence of cross-reactivity since it could be expected that the precision of bacterial enumeration would be dependent on the specificity and brightness of MAb staining. The specificity and coherence of MAb labeling of the complete cell wall is crucial for the automated system, whereas assessors can still accept poorer labeling as indication of presence. The particular advantage of the automated system arises in very dense fields because image processing is very much a field approach, rather than the sequential object approach used by assessors. Hence, high enumeration rates with little interference from time related photobleaching phenomena are achieved. However, both assessor and system experience difficulty in dealing with partially masked or hidden cells and with autofluorescent and detrital material. The problem of false-positive objects is handled by the assessor by 'experience', whereas in these automated analyses relatively simple judgement criteria have been utilized. As shown by Fig. 6 these criteria could not fully prevent counting errors at very low densities (or absence) of true-positive cells. To improve the overall enumeration of dental plaque samples, especially with regard to these false-positive particles that affect the enumeration of bacteria present at very low density, the gradient filter algorithm will probably need to be made more species-specific. Broadly, a gradient filter approach should be useable for all species. However, training periods for each species should result in optimized algorithms, each appropriate for each form. In this way, multiple selective decision rules can be incorporated into each algorithm as opposed to the overall best-fit situation currently implemented.

Previous reports by Kirchman(22) have indicated that, depending on the information content and complexity of an image field, assessors required on average 30 seconds for fields containing between 10–70 organisms. Automated enumeration algorithms described by Sieraki(16) for planktonic

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cells and Xu-van Opstal(38) for eukaryotic cells report on average 6 seconds and 3–10 seconds per viewing field. For comparison, in this work analysis times of about 2 seconds for the gradient filter and 6 seconds for the fast gray morphology (DIC) approaches, which are also relatively independent of organism density, can be viewed as 'competitive'.

The present automated image analysis system was validated by a series of experiments with samples of increasing complexity. Initial system validation was carried out using monodisperse fluorescent polystyrene latex microspheres. Comparison of enumeration results for the most time efficient gradient filter (fluorescence image) and gray morphology (DIC image) algorithms indicates good agreement between the viewing modes and the average assessor. Values for mean differences between assessor and system of 1 to 2 objects for fields exhibiting an object density of 1–1000 objects and in differing spatial patterns was taken as a good indication of the robustness of the methodologies. The approach to the level of agreement was via an iterative learning process, resulting in optimized training set variables. The strength of this approach is the ability to generalize (optimize) it for objects exhibiting differing morphological characteristics.

To confirm that the further design criteria of specificity, equivalency and congruency as defined by Gmür(9) were met, serial dilutions from a master batch of fluorescent beads were enumerated with the automated system. Results taken from repeat aliquots are in acceptable agreement. The data presented in Table 2 show the expected decrease in variability of enumerated totals with increasing sample area studied and total beads counted. Although not investigated thoroughly, observations of the system in operation appeared to indicate that bead number and density variations across the viewing area is the most important factor affecting the uncertainty associated with the enumeration scores. These observations are consistent with similar previous reports.(22) A single, master scanning strategy (16×16 raster pattern) was found to give good indications of bead density over 4–5 orders of magnitude. This again meets an initial design criterion. The scanning strategy could be designed to be auto adjusting, depending on the balance between expected and encountered objects, simply by forming sub-patterns of $m \times n$ design in the master pattern (preferably, m and n are factors of 2). In previous studies, authors detailing enumeration data have generally opted for simpler counting strategies.(39) An in-depth analysis of this enumeration problem has been presented by Kirchman,(22) wherein he describes a cost analysis procedure, which recommends counting a minimum of 400 cells. Future work will investigate these issues more thoroughly, including aspects of a spatial distribution of the cells.

Validation of the automated analysis system with respect to bacterial cultures and biofilm model systems was geared towards utilizing a panel

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of MAbs to perform routine enumeration studies. Example data from the analysis of a pure culture of *S. oralis* show close agreement between the automated system and two assessor's counts. Comparative enumeration of *S. oralis*, *A. naeslundii* and the total number of bacteria in more complex biofilm samples by the automated system and by culture reveal also a very encouraging level of agreement, in spite of comparing in this example (Fig. 5) CFU counts with single cell counts. A severe test of the automated system was experienced with subgingival and interdental plaque. In these cases, detrital material, clumping, low labeling efficiency and false positives objects were all expected to be least controlled. Comparative enumeration results between automated system and a single assessor are in good agreement. In particular, the 'well known' aggregation behavior of *P. gingivalis* and its propensity to shed small antigen-positive vesicles were tackled satisfactorily by the standard gradient filter algorithm. Our system has been challenged in further stringent validation tests (18) using 45 even more complexly structured supragingival samples from an in situ plaque model. In spite of increased difficulties due in particular to virtually indisruptable bacterial clumps, these complementary tests underlined the improved accuracy of this new bacterial enumeration procedure. The technique also proved to be very useful for the quantitative analysis of FISH stained dental plaque samples.(19) In light of the ease with which probes to taxa specific rRNA sequences may be derived, this application of automated image acquisition and analysis is particularly promising.

To obtain maximum enumeration rates for the automated system, investigations were undertaken with the nucleic acid stain SYTOX®. Fluorescence brightness was found to be excellent, fluorescence distribution in individual bacteria was good, except for filaments in which fluorescence was fragmented into several spots. Most encouragingly, the ability of the automated system to deal with the dense bacterial fields encountered during the analysis of SYTOX® stained plaque samples (often >500 bacteria per field) was at a level far beyond which assessors can make realistic estimates. Yet, comparisons of the obtained results with visual readings from more dilute samples or from culture analyses confirmed the data. These results clearly demonstrate that the system can sample high numbers of organisms rapidly with good accuracy and, hence, should meet one of its essential design criteria.

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