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Optical Fiber-Based In Situ Spectroscopy of Pigmented Single Colonies

MARKUS WIGGLI,[1] ROBIN GHOSH,[2] AND REINHARD BACHOFEN[1]*

Institute of Plant Biology and Microbiology, University of Zürich, CH-8008 Zürich,[1] and Laboratory of Bioenergetics, University of Geneva, CH-1254 Jussy-Lullier,[2] Switzerland

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We have adapted a commercially available fiber-optic spectroradiometer with diode array detection to record reflection and absorption spectra from single, 1-mm-diameter bacterial colonies. A careful assessment of the performance of the spectroradiometer for this application is reported. In a model study employing colonies from various phototrophic bacteria, we show that the reflectance spectra are reliable within the range of 450 to 820 nm, whereas the transmission spectra yield accurate peak intensities and absorption maxima from 400 to 900 nm. For screening of populations of about 10^4 colonies, fiber-optic transmission spectroscopy provides an attractive and inexpensive alternative to present techniques based on charge-coupled device imaging technology.

Imaging of optical signals from single colonies of microbial populations is of increasing interest for the screening of novel mutants with unusual phenotypes or properties (1, 2, 4, 8, 12, 14, 15, 18, 22, 26, 27). At present, commercial instruments for this purpose are based mainly on the direct imaging of agar plates using a high-sensitivity CCD camera (1, 26, 27). With such systems, different filtered images are analyzed by calibrated grey scales, and an absorption or fluorescence spectrum is reconstructed by the appropriate software (1, 26, 27). The CCD-based imaging technology has the advantage that thousands of colonies from microbial populations can be analyzed simultaneously. A disadvantage of CCD imaging is that the accuracy and resolution of the individual spectra depend greatly upon the step widths (in practice, filters with a 5- or 10-nm band-pass) of the filters employed.

In many cases, however, it is more appropriate to obtain highly accurate spectra from a small number of colonies (10^4 to 10^5). With the advent of high-resolution diode arrays capable of rapid datum acquisition (millisecond time scale), fiber optics-based spectroradiometers operating in either the reflection or the transmission mode present an interesting and inexpensive alternative to the CCD technology.

In this study, we have examined the performance of a commercially available fiber optics-based spectroradiometer, the LabSpec VNIR-512, in obtaining reflectance and transmittance spectra of single colonies of phototrophic purple bacteria and other pigmented microorganisms using a home-built measurement setup.

Purple bacteria are particularly suitable as a model system since they exhibit several absorption maxima due to bacteriochlorophyll and carotenoid pigments over a wide spectral range (5, 7). Although the application of fiber-optic spectroscopy to turbid samples has been described recently (12, 14), to our knowledge, this is the first study to assess the accuracy of this technique for the screening of single colonies.

(A preliminary report of part of this work has been presented recently [25].)

* Corresponding author. Mailing address: Institute of Plant Biology, University of Zürich, Zollikerstr. 107, CH-8008 Zürich, Switzerland. Phone: 411-385 42 80. Fax: 411-385 42 04. Electronic mail address: bachofen@botinst.unizh.ch.

MATERIALS AND METHODS

Abbreviations. CCD, charge-coupled device; TLC, thin-layer chromatography; I/I_0, the measured light intensity/incident (transmission setup) or reflected (reflection setup) light intensity ratio; S/N, signal-to-noise ratio; and LHI, light-harvesting complex I.

Growth of bacteria. *Rhodospirillum rubrum* S1, *Rhodobacter sphaeroides* 158 and 2340, and *Rhodocyclus tenuis* were obtained from the German Collection of Microorganisms (Braunschweig, Germany). Tn*5* mutants of *R. rubrum* were obtained from our in-house collection. All bacterial cultures were grown on Sistrom minimal medium A (19) without Casamino Acids (6) and containing 1.5% agar. Cultures were grown photoheterotrophically in an anaerobic jar under variable light intensities. Agar plates containing single colonies of *Streptomyces coelicolor* A3(2) (strain MT1110) and *Chlamydomonas reinhardtii* were kind gifts of C. Thompson (Biocenter, University of Basel) and R. Strasser (University of Geneva), respectively.

Measurement of transmittance and reflectance spectra with the LabSpec spectroradiometer. A LabSpec VNIR-512 general-purpose spectroradiometer (Analytical Spectral Devices, Inc., Boulder, Co.) was employed in home-built instrumental configurations for both absorbance and reflectance spectroscopy (Fig. 1). In both configurations, light emitted by a tungsten bulb (Bellaphot 64607; OSRAM, Munich, Germany), fed by a tunable, stabilized rectifier (model L15/8BE; Automation + Elektronik, Volketswil, Switzerland), is decreased in heat radiation by passage through a water-filled chamber and thereafter directed into one or two waveguides (GC 600/750, 25 m; Fujikura Ltd., Tokyo, Japan). After exiting, the light interacts with the sample and is partially collected by an optical fiber which guides it to the LabSpec spectroradiometer, from which it is split into spectral components by a holographic grating and analyzed by a photodiode array detector. The spectral resolution is nominally 3 nm, and the sampling interval is 1.4 nm (Analytical Spectral Devices, technical data). The viewing angle of the collecting fiber and the illuminating angle are 25 and 28°, respectively. Incident photons are integrated by 512 detector elements, converted to voltages, and digitized by a 16-bit analog-to-digital converter. The digital data are transferred to a personal computer (3200SX; Toshiba, Tokyo, Japan) using direct memory access. Spectra are recorded as digital numbers (raw data, instrumental characteristics included) for further processing. The software which controls the functions of the spectroradiometer provides real-time visualization of the raw signal or the intensity ratio I/I_0. The control software (Analytical Spectral Devices, Inc.) offers several tools for refinement of the measurements: (i) correction of the signal for the dark current, (ii) repeated doubling of the integration time (17 ms $\cdot$ 2^n) to maximize the S/N by allowing the desired raw signal to approach saturation, and (iii) averaging of spectra to increase the S/N. In addition, instead of use of digital numbers (16-bit range, 2^{16} = 65,536 for every channel), a reference spectrum can be recorded, which is set to 100% and with which the ratios of all further spectra are compared. In this way, all characteristics of the instrument are cancelled out. Such a spectrum is called white reference, since it defines a white color (100%) with respect to a specific illumination and is normally recorded from a special reference material in a given geometrical setup in reflectance mode. In this study, we tested Teflon, white paper, and TLC silica gel plates (Silica Gel 60; Merck, Darmstadt, Germany). In transmission mode, the white reference defines the baseline (I_0). To achieve the highest degree of accuracy, all types of spectra (raw, spectrum ratio I/I_0, dark-

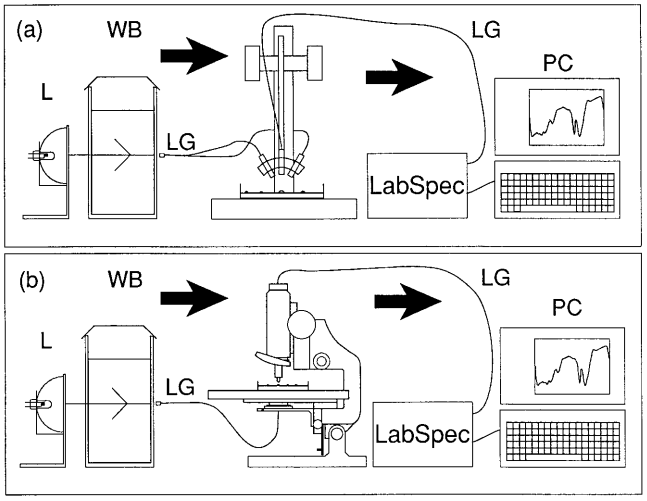

FIG. 1. Reflectance (a) and transmittance (b) configurations of the LabSpec spectroradiometer for the measurement of single colonies. The light source (L), water bath heat filter (WB), light guides (LG), and personal computer (PC) are indicated.

current corrections, and white references) can be averaged. Dark-current correction and white reference measurements are stored for each integration time.

An important consideration in obtaining reliable absorption or reflection spectra with a high S/N is the wavelength dependence of the light source. In our setup, we have employed a tungsten light source at different light intensities, corresponding to different applied voltages. The dependence of the measured raw signal upon wavelength shows large variations (Wien's Displacement Law) in the region of 400 to 600 nm at different applied voltages, thus causing large differences in the S/N of the final spectra from this region (Fig. 2a).

Stored binary data (occupying only 2,532 bytes per spectrum) are then converted into ASCII (up to 999 files per batch). For further data processing and visualization, we have routinely employed SigmaPlot (Jandel Scientific). The ratio of spectra (I/I_0) can be transformed to absorption spectra by using the relation $A = \log_{10} (I_0/I)$. Larger algorithms to search local extrema and to perform additional averaging, baseline correction, normalization, smoothing, sorting, and other data processing were implemented, using the macro programming language of SigmaPlot. Since visual examination is possible at every step, outliers and other problems can be detected, keeping the algorithms quite small. These datum manipulation algorithms will be provided on request.

Reflection or absorption spectra were obtained from single colonies as follows. (i) In the reflection mode (Fig. 1a), two fibers, each 30° to the optical axis, the latter defined by the collecting waveguide, form a spot of light at a 2.5-mm distance (the measuring head, which was crafted in the institute's workshop, is mounted on a holder [B. Braun, models 1204 no. 772 and 1206 no. 504; Apparatebau, Melsungen, Germany]). (ii) In the transmission configuration (Fig. 1b), the light-emitting and the light-collecting fiber define the optical axis, which is guaranteed by a microscope positioning table (Winkel-Zeiss, Göttingen, Germany) (insets were made in the institute's workshop). The holders provide exact positioning along the optical axis in both cases.

RESULTS AND DISCUSSION

Optimization of the instrumental setup for measuring reflection or absorption spectra of single colonies. In the reflectance setup, the choice of the white reference is important for the accuracy of the final spectrum. Silica gel plates, Teflon, and white paper were therefore tested for their performance as white reference materials. Of these, the TLC silica gel plates proved to be excellent, showing a high reflectance over the complete usable range (Fig. 2b). Unexpectedly, Teflon showed a weak reflection over the whole range, decreasing towards longer wavelengths and dropping below 20% of the TLC plate reflectance above 700 nm. The reflectance from white paper was superior to that of the TLC plate above 630 nm but shows a significant drop below 420 nm.

Reflectance measurements of colonies on agar medium were routinely performed after placement of the petri dish on a TLC plate. However, the reflectance of agar in this configuration was only a small percentage of that of a TLC plate alone (Fig. 2b), so data collection was usually performed by adjusting the white reference signal to the agar-TLC background. The use of agar-TLC plates as a white reference assures that the reference reflectance signal is not much larger than the reflectance signal of the sample, which allows the use of a longer integration time for both spectra (to optimize the S/N) without the saturation level of detection being reached. A possible complication is that some species (e.g., *S. coelicolor*) may leak substances into the medium in the vicinity of a colony, thus affecting the white reference signal (data not shown). In such cases, it is advisable to measure white reference signals at various distances from the target colony.

As the sensitivity of the detector is an important factor for obtaining reliable spectra with high S/N ratios, the raw signal

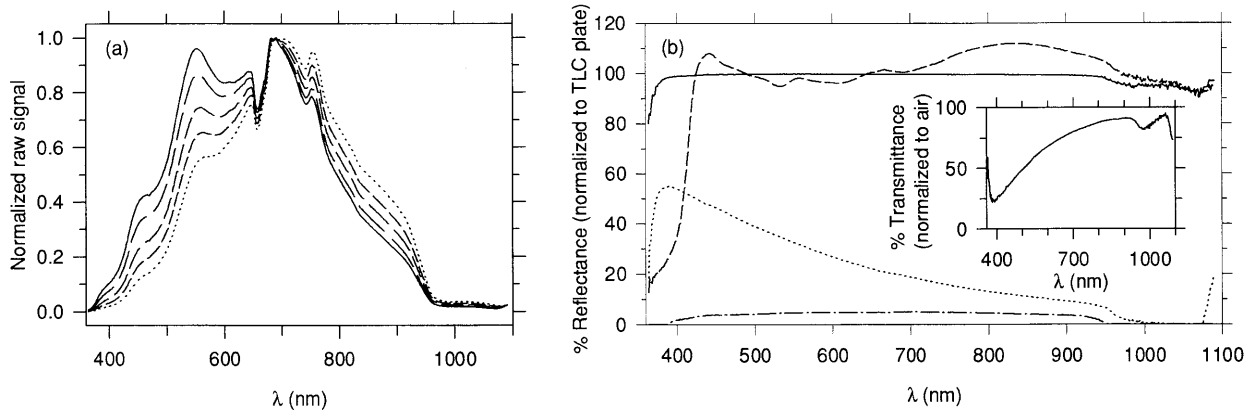

FIG. 2. (a) Variation of the relative intensities of the raw signal at different wavelengths in response to changes in the intensity of the tungsten illumination source by application of different voltages (8, 6.5, 5, 4, and 3.2 V, solid to increasingly dissolved lines, respectively). The normalized raw signals were obtained by setting the individual maxima to 1.0. The integration times (17 to 272 ms) were chosen to approach the full range (16 bit) of the analog-to-digital converter. The prominent local decrease around 660 nm is due to the physical characteristics of the grating. (b) White reference profiles obtained using the reflectance configuration. Reflectance signals were obtained from the following white reference materials: Teflon (dotted line), white paper (dashed line), and a silica gel TLC plate (solid line), used as a reference. The reflectance signal of an agar-filled petri dish placed on a TLC plate (dashed-dotted line) is also shown. Values above 900 nm are unreliable, as the S/N in that region is very low. (Inset) Transmission profile obtained with an agar plate, illustrating the high level of transmission observed between 800 and 900 nm in this setup.

was analyzed by using a calibrated tungsten lamp (National Institute of Standards and Technology reference, which was kindly made available to us by the World Radiation Center in Davos, Switzerland) which exhibits a continuous black body spectrum with a single maximum flanked by a steep decrease to shorter wavelengths and with a long tail towards longer wavelengths. The analysis showed that the sensitivity is high between 500 and 800 nm but drops significantly outside this range. The raw spectrum was comparable to those shown in Fig. 2a (see reference 25), except for the more prominent decreases in raw signal above 950 nm caused by heat filtering in the latter setup.

Reflectance and absorbance spectroscopy of single colonies of pigmented microorganisms. We have tested the performance of the transmission and reflection setups using the phototrophic bacterium *R. rubrum* as a model system. Reflection spectra for colonies approximately 1 mm in diameter showed a high degree of reproducibility in the range of 450 to 820 nm (see Fig. 3 and 5) and gave peak maxima and relative intensities identical to those of absorption spectra taken for colonies resuspended in medium by using a conventional spectrophotometer (Uvikon 860; Kontron Instruments, Zürich, Switzerland) with a second sample position close to the photomultiplier for the measurement of turbid samples. In this range, the accuracy of the peak maxima was ±1 nm (Table 1). However, at the extremes of the practical wavelength range, large deviations of the expected absorption spectra (transformed from reflectance data) were observed with major signal losses and spurious shifts of peak maxima (up to 5 to 10 nm from the maxima measured in the transmission setup) at both extremes of the wavelength scale towards the center of the usable range. Extensive adjustments of the reflectance setup as well as attempts to improve spectra by measuring different-size colonies failed. By contrast, data obtained in the transmission setup reliably yielded spectra comparable to those obtained for resuspended colonies by conventional absorption spectroscopy. We also confirmed that the transmission mode spectra exhibited relative peak maxima of known optical transitions (Table 1).

Inspection of the transmission spectra of the wild-type *R. rubrum* S1 (Fig. 3a) shows that the absorption maxima due to the carotenoids (485, 514, and 549 nm) (23) and the bacteriochlorophylls (588, 803, and 884 nm) (20) of the reaction center and the LHI complex (7, 20) can be determined with a high level of precision ($<\pm 1$ nm) (Table 1). In addition, the relative intensities of the various pigments are well reproduced with the transmission setup. The utility of this setup for the screening of mutants is indicated in Fig. 3b to d. Figure 3b shows spectra from colonies of a carotenoidless mutant, *R. rubrum* ST2 (24). In this case, the carotenoid peaks are missing and the near infrared maximum of the LHI complex is shifted to 873 nm, as expected from conventional spectra. Figure 3c shows a spectrum obtained from colonies of a Tn*5* mutant of *R. rubrum* with a lesion in the *crtX* gene encoding rhodopin 3,4-desaturase of the carotenoid biosynthetic pathway. In this mutant, a modified carotenoid is incorporated into the LHI complex and reaction center (9). The chemical modifications in the mutant carotenoid produce an absorbance shift of approximately 20 nm for all of the carotenoid maxima, whereas the maxima due to bacteriochlorophyll remain unchanged in comparison with those of the wild type. This is easily observed with the transmission setup. Figure 3d shows results for a Tn*5* mutant of *R. rubrum* with a lesion in the *bchXYZ* genes of the bacteriochlorophyll biosynthesis pathway which leads to the accumulation of the precursor bacteriochlorophyllide (16, 17). A conventional spectrum of the bacteriochlorophyllide-accumulating mutant shows a weak absorption maximum at 665 nm, with smaller absorption maxima at 510 and 540 nm. These were also observed in the transmission setup. The high-scattering background observed for the transmission spectrum (and also in the conventional spectrum) of this strain is typical for mutants of *R. rubrum* with perturbations in the biogenesis of intact photosynthetic membranes (data not shown). This may be characteristic of photosynthetic bacteria because of the long-range ordering of their photopigments (see below). These data confirm the suitability of fiber-optic spectroscopy as a rapid screening method for detection of small changes of peak absorption maxima or hyper- or hypochromic effects.

The results obtained with *R. rubrum* were confirmed for the phototrophic bacteria *Rhodobacter sphaeroides* and *Rhodocyclus tenuis*, as well as the pigmented bacterium *S. coelicolor* and the green alga *C. reinhardtii* (Fig. 4). Transmission mode spectra obtained from single colonies of *Rhodobacter sphaeroides* 158 (wild type), carotenoidless mutant *Rhodobacter sphaeroides* 2340, and *Rhodocyclus tenuis* were identical to those obtained with a conventional spectrophotometer (Fig. 4a to c) and allowed the two strains of *Rhodobacter sphaeroides* to be

TABLE 1. Comparison of absorption maxima of phototrophic bacteria obtained by different measurement setups[a]

Organism	Transmission			Conventional λ_{max}	Reference(s)
	Mean λ_{max}	SD	No. of colonies		
R. rubrum					
S1 (wild type)	485*	1.77	10	485*	6, 20, 23
	513*	0.83	19	514*	
	547*	0.67	19	549*	
	587	0.22	19	588	
	759	3.66	19		
	799	0.78	19	803	
	881	0.79	19	884	
ST2 (carotenoidless)	588	1.49	13	587	24
	760	2.08	7		
	799	1.08	13	801	
	873	0.85	13	874	
ST4 (*crtX*::Tn*5*)	489*	1.35	24	488*	9
	523*	1.67	21	523*	
	589	0.83	24	590	
	797	1.51	24	802	
	882	0.72	24	881	
ST3 (*bchXYZ*::Tn*5*)	672	0.00	3	665	16, 17
Rhodobacter sphaeroides					
158 (wild type)	450*	2.58	22	450*	
	479*	0.87	22	476*	
	511*	0.97	22	510*	
	588	0.67	22	588	
	799	0.64	22	800	
	851	0.92	22	852	
2340 (carotenoidless)	587	1.09	3	591	
	798	1.67	3	799	
	860	0.00	3	862	
Rhodocyclus tenuis (wild type)	465*	2.16	5	464*	
	491*	1.10	7	492*	
	592	1.23	9	590	
	796	1.53	9	800	
	872	1.66	9	871	

[a] The major absorption maxima arising from carotenoids (indicated with asterisks) and bacteriochlorophyll are listed. For the characteristic absorption maxima cited in this study, we have generally used those determined from conventional spectrophotometry. The exact positions of these maxima can vary as much as ±1 nm when measured with different instruments.

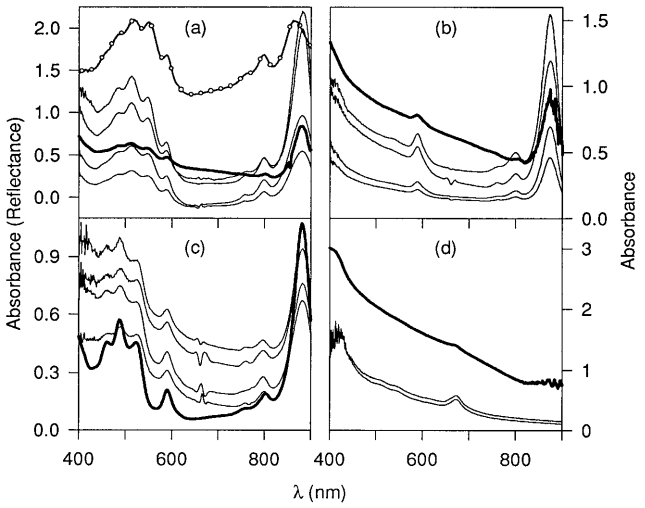

FIG. 3. Spectra of wild-type *R. rubrum* and mutants of *R. rubrum* recorded in transmission (thin lines) and reflection (circled lines) modes and compared with conventional spectrophotometry (thick lines). (a) *R. rubrum* S1 (wild type); (b) *R. rubrum* ST2, a carotenoidless mutant; (c) *R. rubrum* ST4, containing a lesion in the rhodopin 3,4-desaturase of the carotenoid biosynthesis pathway (9); (d) *R. rubrum* ST3, a mutant with a Tn*5* insertion in the *bchXYZ* genes of the bacteriochlorophyll biosynthesis pathway (17). The conventional spectra from liquid cultures were recorded using 1-cm-path-length cuvettes placed at the sample position for turbid samples close to the photomultiplier in a Uvikon 860 spectrophotometer. For all transmission and reflection spectra shown, the voltage of the tungsten illumination source was 5 V.

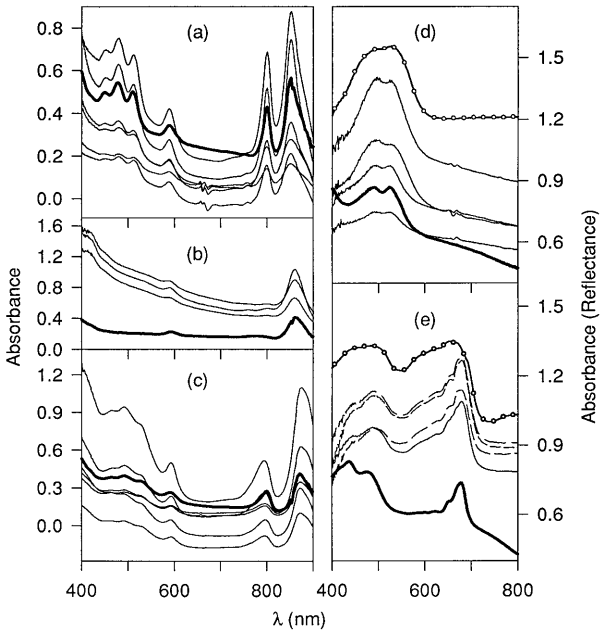

FIG. 4. Spectra of a selection of pigmented microorganisms recorded in transmission mode (thin lines) or reflection mode (circled lines) and compared with conventional spectrophotometry (thick lines). (a) *Rhodobacter sphaeroides* 158 (wild type); (b) *Rhodobacter sphaeroides* 2340, a carotenoidless mutant; (c) *Rhodocyclus tenuis*; (d) *S. coelicolor* A3(2) (strain MT1110) (wild type); (e) *C. reinhardtii*. In this figure, the relative intensities of individual spectra were adjusted to facilitate visual inspection. For most spectra of *C. reinhardtii*, the illumination source voltage was raised to 8 V to increase the S/N around 400 nm. For the same reason, 16 averages were taken before storage for all spectra.

easily identified. In all cases, the positions of the peak maxima as well as the relative intensities corresponding to the carotenoids and LHI complexes were exactly reproduced (Table 1). The performance of the spectroradiometer in the near infrared region of the spectrum is well illustrated by the appearance of a slight shoulder at approximately 875 nm observed for both *Rhodocyclus tenuis* and *Rhodobacter sphaeroides* 158. This shoulder corresponds to the overlapping peak maxima of LHI (875 nm) and LHII (850 nm) complexes (5).

Figure 4d shows transmission spectra obtained from several colonies of the gram-positive bacterium *S. coelicolor* A3(2) (wild type, strain MT1110). The twin overlapping maxima at 492 and 524 nm are due to colored secondary metabolites (undecylprodigiosin [red] and actinorhodin [violet] [21]) produced during development of the colonies. Inspection of this panel shows that the relative intensities of the two peaks vary. We believe that this variation is due not to the measurement setup but to the difficulty of positioning the measuring beam at an area of the colony which corresponds to the same developmental phase in all cases. Indeed, measurement of spectra at increasing radii from the center of the same colony yields differing relative absorption maxima (data not shown), and it is known that secondary metabolite production in *S. coelicolor* varies with colony morphology (21). Measurement of colonies of *S. coelicolor* with the reflection setup also proved to be reliable, although larger variations than for the transmission spectra were observed. Figure 4e shows transmission, reflectance, and conventional spectra for colonies of *C. reinhardtii*. Although the transmission spectra strongly resembled conventional spectra in the positions of the absorption maxima at 680 nm (because of photosystem II and its associated antenna) and the shoulder at 645 nm (mainly antenna containing chlorophyll *b*), detailed inspection seemed to show minor additional components within this region. We are not certain whether these differences represent developmental variations between different colonies of *C. reinhardtii* or are caused by the appearance of fluorescence due to the relatively high intensity of the measuring beam. The latter would be indicated by the large distortions observed for the reflection spectrum.

For reflection spectroscopy, the quality of the reflection spectra varied widely with the size of the colony measured; optimal spectra were obtained with 1-mm-diameter colonies. However, even under conditions in which small colonies were measured, the quality of the reflection spectra observed varied widely with the species used. Thus, reflection spectra obtained for *R. rubrum* S1 and *S. coelicolor* were often reliable [standard deviations were typically ± (2 to 4) nm between 450 and 820 nm] in comparison with the transmission spectra, whereas those obtained for *C. reinhardtii* appear severely distorted. By contrast, transmission mode spectra were relatively insensitive to colony size, although the positioning of the measuring beam to the center of the colony was essential for obtaining spectra with a high dynamic range (Fig. 5). However, as shown in Fig. 5, measurement at the center of the colony was not essential for obtaining reliable spectra. Figure 5 also shows that the signal is nearly zero when the optical axis of the measuring beam was placed at the border of the colony. This facilitates the reliable measurement of closely spaced colonies.

Using a reflectance setup, we have also been able to obtain absorption spectra [log (1/reflectance)] from colored extracts chromatographed on TLC plates or from cells collected on filters (3), with no shifts at the extreme ends of the usable range. We conclude, therefore, that the observed spectral distortions obtained even from small single colonies by use of reflectance measurements probably arise from the unusual optical properties of highly curved photosynthetic membranes containing large amounts of tightly packed chromophores (10,

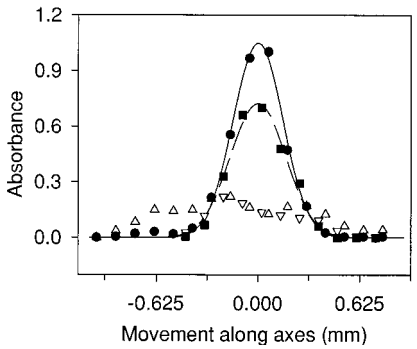

FIG. 5. Influence of positioning of the measuring beam upon the spectral dynamic range obtained for a single colony of *R. rubrum* (diameter, 0.625 mm). The center of the colony was placed on an axis by eye, along which a first series of spectra were recorded at increasing distances from the origin. Then the petri dish was turned 90°, and a second series was taken. The starting points (on the left) outside the region occupied by the colony were chosen arbitrarily. Maxima at 881.4 nm were corrected by subtracting the value of the absorption at 700.7 nm (open triangles), at which no specific absorption is observed. The difference absorption profiles obtained for the two orthogonal measurement axes (closed circles and closed squares, respectively) were fitted with Gaussian curves with their positions of the maxima set to zero, defining the center of the colony. The different maximal amplitudes of the orthogonal measurements are due to visual inaccuracy in aligning the measurement axes and the center of the colony.

11) which may be enhanced by the geometrical shape of the colonies. A further indication of the anomalous optical properties of single colonies of phototrophic bacteria is that with both transmission and reflectance setups, we were able to record more light reaching the collecting fiber during colony measurement than when the agar surface alone was scanned. This effect may also be attributed to the enhancement of light scattering by highly electronically coupled arrays of chromophores at the absorption maxima (resonance light scattering) reported recently (13). For mutants in which the chromophoric order of the photosynthetic apparatus is disturbed, single colonies usually appear to be more opaque than the wild type, and the spectra obtained show a corresponding high degree of scatter (e.g., *R. rubrum* ST3 [Fig. 3c]).

In this study, we have shown that rapid fiber-optic spectroscopy of single bacterial colonies is capable of yielding highly accurate reflectance and absorbance spectra. The accuracy of the spectra obtained is sufficient to determine small changes (± 2 nm) in peak maxima which are in the practical range for many screening applications. In our manual setup, it is possible to measure at least 500 colonies per day. As the spectra are integrated over 17 ms and repeated doublings of 17 nm, screening of a large number of colonies is feasible, particularly when the colony positioning is automated. In addition, the basic setup is inexpensive compared with CCD-based screening technologies. We are currently refining the transmission setup for automated screening of large populations of microbial colonies.

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