On-Line 3-Dimensional Confocal Imaging In Vivo

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PURPOSE. In vivo confocal microscopy through focusing (CMTF) can provide a 3-D stack of high-resolution corneal images and allows objective measurements of corneal sublayer thickness and backscattering. However, current systems require time-consuming off-line image processing and analysis on multiple software platforms. Furthermore, there is a trade off between the CMTF speed and measurement precision. The purpose of this study was to develop a novel on-line system for in vivo corneal imaging and analysis that overcomes these limitations.

METHODS. A tandem scanning confocal microscope (TSCM) was used for corneal imaging. The TSCM video camera was interfaced directly to a PC image acquisition board to implement real-time digitization. Software was developed to allow in vivo 2-D imaging, CMTF image acquisition, interactive 3-D reconstruction, and analysis of CMTF data to be performed on line in a single user-friendly environment. A procedure was also incorporated to separate the odd/even video fields, thereby doubling the CMTF sampling rate and theoretically improving the precision of CMTF thickness measurements by a factor of two.

RESULTS. In vivo corneal examinations of a normal human and a photorefractive keratectomy patient are presented to demonstrate the capabilities of the new system. Improvements in the convenience, speed, and functionality of in vivo CMTF image acquisition, display, and analysis are demonstrated.

CONCLUSIONS. This is the first full-featured software package designed for in vivo TSCM imaging of the cornea, which performs both 2-D and 3-D image acquisition, display, and processing as well as CMTF analysis. The use of a PC platform and incorporation of easy to use, on line, and interactive features should help to improve the clinical utility of this technology. (Invest Ophthalmol Vis Sci. 2000;41:2945–2953)

Tandem scanning confocal microscopy (TSCM), with its unique optical sectioning ability,1 is used for in vivo corneal imaging of both laboratory animals and human patients. Published research applications of confocal microscopy include quantification of changes in epithelial morphology after contact lens wear,2 assessment of corneal wound healing,3,4 and measurement of the effects of ocular irritation5,6 (for review, see Ref. 7). Clinical applications include the early detection and diagnosis of corneal infections,8,9 evaluation of corneal wound healing after excimer laser eye surgery,10–12 and assessment of the effect of contact lens oxygen transmissibility on the corneal epithelium13,14 (for review see Refs. 15, 16). TSCM image acquisition and data processing systems have experienced several design improvements since the instrument was first produced. Our laboratory developed the first real-time in vivo image acquisition system for digitizing high-magnification 2-dimensional (2-D) TSCM images of corneal cells using a real-time digital disk.17 The subsequent development of an objective lens in which the z-axis position of the focal plane could be moved in calibrated increments allowed sequential sections of the in vivo cornea to be obtained. This allowed high-magnification, high-resolution, 3-dimensional (3-D) reconstructions of corneal cells to be generated.18

In 1995, Confocal microscopy through focusing (CMTF) was developed in our laboratory using a Unix graphic workstation (INDY; Silicon Graphics, Mountain View, CA) and has since been used for objectively measuring corneal sublayer thickness and performing 3-D reconstruction from a stack of 2-D CMTF images using the ANALYZE image analysis software package (Mayo Medical Ventures, Rochester, MN).19 One shortcoming of this approach is that CMTF scanning is performed separately from image acquisition, processing, and data analysis. During an examination, video images are recorded on the videotape, and usually no further information is collected (on-line scanning, off-line processing). This offers limited feedback information at the time of examination, and off-line digitizing and analysis of images from videotape is very time-consuming. Another limitation of many existing systems is that they use a Unix workstation or a mini-computer interfaced to a specialized image acquisition device, which tends to be expensive and unfamiliar, thereby limiting widespread appli-
cation and use. More recently, we developed a PC-based image acquisition system for off-line TSCM imaging digitizing from videotape using a commercial PC image acquisition board.\(^{20}\) However, the INDY workstation was still used for 3-D reconstruction and display. Although the system hardware platform was simplified, TSCM imaging, especially CMTF analysis, remains a labor-intensive process.

In this article, we describe a new PC-based TSCM system featuring integrated supporting software to provide on-line scanning, processing, visualization, and analysis. The system is powerful and convenient and has a concise design, and should thus improve the usefulness of in vivo confocal microscopy as a tool for ophthalmic research studies and clinical patient diagnoses. The major new features of the system include real-time image digitizing, on-line temporal moving average for 2-D imaging, on-line CMTF acquisition and data analysis, on-line interactive 3-D reconstruction and display, and doubling of the CMTF sampling rate.

**METHODS**

**System Hardware Configuration**

Figure 1 illustrates the system hardware configuration. Previously, the system was composed of five functional subsystems: (1) A tandem scanning confocal microscope (Tandem Scanning Corp., Reston, VA) with a 24 ×, 0.6 NA applanating objective with a variable working distance (0–1.5 mm);\(^{18,20}\) (2) objective lens motion control: a linear actuator in the microscope and its controller (Model 18011; Oriel Instruments, Stratford, CT); (3) image acquisition and data processing: a low light level video camera (Dage VE1000; MTI, Michigan City, IN) interfaced to a high performance Personal Computer (Pentium II, 400MHz/384Mbyte RAM; Dell Computer, Austin, TX) with a plug-in image acquisition board (DT3152; Data Translation, Marlboro, MA); (4) video recording: a time code generator (Model F30; Fast Forward Video, Irvine, CA) and a video monitor connected to a Super VHS recorder; and (5) power supply. In the new system, which is on the left of the dashed line in Figure 1, the camera output is interfaced directly to the image acquisition board. By using the new customized PC software to synchronize the processes of focal plane movement and real-time image acquisition, on-line scanning and processing is achieved. Thus, the video recording subsystem (right of the dashed line in Fig. 1) is now used only as a video backup subsystem. Furthermore, the time code generator is not needed for on-line imaging, because the computer imprints the scan time and focal plane depth directly onto digitized images (see On-line CMTF, below).

**System Operation and User Interface**

In vivo confocal data from two patients are presented to demonstrate the features of the new system; both patients gave informed consent and the research adhered to the Declaration of Helsinki. To perform an in vivo corneal examination, the operator first needs to align the objective tip with the central corneal surface using the joystick and deck elevator. A drop of 2.5% methylcellulose solution is placed on the tip of the objective lens to serve as a liquid cushion to help stabilize the cornea and to reduce bright surface reflections from the lens tip. The tip of the lens is lightly applanated to the cornea, thus minimizing z-axis drift. All other imaging operations run under control of the newly developed user-friendly Windows program, called TSCM2.0. This all-in-one software integrates the functions of focal plane control, image acquisition and processing, CMTF data analysis, and file management on line.

The major user interfaces of the TSCM2.0 program are shown in Figures 2 and 3. Briefly, the Windows interface is split into three functional areas: focal plane control, image processing and display, and intensity curve manipulation, each of which has corresponding command buttons. By using the mouse or keyboard to press these command buttons, the user can trigger actions such as changing the focal plane position; changing the lens speed; displaying a live image; launching a CMTF scan; displaying or rotating the 3-D reconstructed volume; or calculating corneal thickness or haze. To make the software more flexible, the application settings are user-selectable through a Windows dialog box (Fig. 2). For example, the user can determine: the number of images in a CMTF scan (i.e., the depth of the CMTF scan); whether or not to use double sampling rate; the interval over which to perform a temporal moving average; and how many neighboring pixels to use to smooth side view images and 3-D surface images.

**Real-Time Image Acquisition and Display**

To implement both on-line scanning and on-line processing, images must be digitized in real time. This is made possible by using an 8-bit black/white PC image acquisition board, DT3152 (Fig. 1). Because the camera output is fed directly to the PC image acquisition board, the program can display a live digital image on the computer screen (Fig. 2). Using “passthrough” mode, the board digitizes the analog video signal and stores the data in an image buffer within its device memory (a part of the PC system RAM allocated for the board use). The image buffer is mapped to the screen buffer so that both digitizing and display occur in “real time” (30 frames/sec). The live image has a frame size of 640 × 480 pixels and can be captured (freeze

![Figure 1. System hardware configuration. The camera output is interfaced directly to the PC image acquisition board DT3152. The video recording subsystem (right of the dashed line) is used only for video backup.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932909/ on 04/02/2017)
frame) and saved in TIFF format on line. This is useful for the capture and archiving of corneal images for quantitative analysis (e.g., measurement of epithelial cell size and density).

Because of the unique optical path of the tandem scanning confocal microscope, only a very small fraction of light contributes to the detected image, which results in a generally low signal-to-noise ratio (SNR) of the image data. Furthermore, because of unexpected eye movements relative to the objective tip, single images are often blurred when captured. Therefore, an “acquire multiple” mode can be used to capture 60 sequential live images (total 2 seconds' duration) at the same focal depth so that the user can discriminate and select a single, nonblurred image. The process of selecting a good image from the sequence is performed interactively by moving the mouse cursor to scroll through the images.

![Diagram](https://example.com/diagram.png)

**Figure 2.** The user interface of the TSCM2.0 program for displaying a live digital image and the dialog box for changing program settings. To make the software more flexible, most of the application settings are user-selectable.

Optionally, the user can use a new temporal moving average operation to improve the SNR of the images within the sequence. Although eye movements normally occur during acquisition of the sequence, there are typically stationary periods in the sequence between movements. Temporal averaging over these stationary periods will result in an image with improved SNR and preserved edges. The temporal moving average can be expressed as:

\[
g_t(x, y) = \frac{1}{2m + 1} \sum_{k=-m}^{m} f_k(x, y),
\]

where \( m < t < N - m - 1 \), \( n = 2m + 1 \).
where $N$ is the total number of frames in the sequence (currently 60), $n$ is the user-selectable width of the averaging window (i.e., number of frames to be averaged), and $f(x, y)$ is the original image sequence and $g_i(x, y)$ is the averaged image sequence. Noise is reduced by a factor of $\sqrt{n}$ using this procedure. In TSCM2.0, after the averaged images are calculated they are written into the original sequence of buffers so that the user can scroll through them quickly using the mouse cursor to select the best quality image.

**On-line CMTF**

CMTF is an operation of rapidly moving the focal plane through the entire cornea at high speed while images are acquired ($320 \times 240$ pixels), resulting in a stack of 2-D images. A $z$-axis intensity profile is then generated by calculating the average pixel intensity in a central region (user-selectable, typically $100 \times 100$ pixels) of each image and plotting versus $z$-depth.\textsuperscript{19} The intensity curve provides quantitative data about the depth, thickness, and backscattering of corneal sublayers. As the user moves a cursor along the intensity curve, the corresponding images are displayed. In this way, the user can identify images of interest and record their exact $z$-axis positions. Previously, CMTF images were first recorded on a videotape and later were digitized off line from the videotape to create the intensity curve using a specialized image acquisition program.\textsuperscript{19} This process can be very time consuming as the number of exams grows larger.

On-line CMTF acquires corneal images to the device memory of the DT3152 board directly from the video camera while the focal plane is passing through the cornea. When a CMTF command is launched on the screen, a signal is sent to the Oriel 18011 controller via the serial port to start the focal plane movement. Image acquisition is then simultaneously started by sending an “acquire multiple” command to the DT3152, which digitizes sequential images to a sequence of buffers in device memory. We are currently using the “synchronous acquire” mode, which ensures that no video frames are skipped during the acquisition. After the user-selected number of images has been digitized (typically 400 for a normal cornea), the image acquisition process is terminated and a signal is sent to the Oriel controller to halt the lens movement.

![Figure 3. On-line CMTF scan and data processing of a normal human cornea in vivo. The measured epithelial thickness, stromal thickness, and corneal thickness were 62.5, 504.4, and 566.9 $\mu$m, respectively. 3-D reconstruction was performed interactively on line.](image-url)
Immediately after an on-line CMTF scan ceases, the intensity curve is generated instantly and displayed in a “curve pane” on the right half of the PC screen (Fig. 3). The user can then mark the positions in the curve corresponding to the layers of interest to obtain thickness information on line. Intensity curve data, along with the CMTF settings and measurement information, can be saved to a text file. This file can be exported to other software such as Microsoft Excel when necessary. The intensity curve file and the image stack file thus provide uniquely important, although not complete, physical information about the cornea. A speed controllable movie has also been incorporated to replay the CMTF scan digitally.

Time and depth labeling of each CMTF image in the stack is very important for thickness measurements and for localizing structures within the 3-D volume. We initially developed a depth encoding system (DES) for this purpose in a previous TSCM system. In that system, because of the time delay in reading lens position from the controller, the depth imprinted in the video image through the F30 time code generator was somewhat delayed. For on-line CMTF, we have developed a more efficient method for time and depth labeling. In the TSCM2.0 program, the time and depth are imprinted into the top left corner of each image using an “OR” operation immediately after the completion of a CMTF scan (Fig. 3). Because the system keeps track of when and where the CMTF starts and the focusing speed it uses, the time and depth of each image in the CMTF sequence can be calculated and labeled accurately. Time and depth labeling of captured live 2-D images can be accomplished using the same method. Thus, the new system can operate without the time code generator; further simplifying system design and reducing the cost.

Using the standard frame rate (30 frames/sec) and a typical lens speed of 160 µm/sec, the average CMTF focal plane speed is approximately 64 µm/sec. Thus, a 400-image sequence will scan 853 µm, which more than covers the average thickness of human cornea. Images are subsampled (320 × 240 pixels), so that a stack of 400 images uses 30 Mbytes of RAM. The image stack can be saved to disc, and loaded back into the TSCM2.0 software environment whenever it is desirable to re-review, display, or further analyze the scan. We have even developed a version of the program that runs without the digitizing board and can be used on a lap-top computer. Of course, by disabling the lens control commands and accepting input from videotape, the program can also perform off-line CMTF acquisition and data analysis if necessary.

Interactive 3-D Reconstruction for Visualization

The on-line acquired 2-D CMTF image stack (coronal views) in the device memory can also be used to create side views and generate 3-D reconstructions (Fig. 3). The side view from the x direction (sagittal view or y-z slice) is a reconstructed 2-D image that consists of a column from each image in the sequence at a user-selected x-axis position. The side view from the y direction (transverse view or x-z slice) is a reconstructed 2-D image that consists of a row from each image in the sequence at a user-selected y-axis position.

An oblique projection is used to create the surface image of the 3-D reconstructed volume. The front view of the volume is an x-z slice. The right side of the volume is formed by taking every other row of a y-z slice and projecting upward 45°. The top view of the volume is created by picking every other row of the current 2-D image in the sequence (selected using the cursor) and projecting to the right 45°. These three surface images are combined seamlessly to create simple 3-D reconstruction. To smooth the noise, the side views and the surface images are averaged with their neighboring pixels. For the convenience of visual inspection, the image sequence, its side views, and 3-D projection are displayed together (Fig. 3). In addition, the 3-D reconstructed volume can be rotated along the x, y, or z-axis.

Similar to the x-ray sectioning of a computerized tomography (CT) scan, reconstructed volume slicing has been incorporated so that a group of 2-D images can be displayed in a floating desktop window frame by frame with a user-selectable spatial distance (not shown). Volume slicing can be done in the x, y, or z direction. This is useful when it is necessary to check the image transitions of a corneal structure two dimensionally.

Doubling of CMTF Sampling Rate

The distance between two adjacent CMTF images ∆d, i.e., the sampling interval of the z-axis intensity curve, is determined by the focal plane speed, v_p, and the frame rate, r, which is

\[ ∆d = \frac{v_p}{r}. \]

The focal plane speed can be precisely calculated from the lens movement speed by a third-order polynomial equation provided by the manufacturer, which is slightly nonlinear. When using the standard frame rate and a lens speed of 160 µm/sec, the average focal plane speed is approximately 64 µm/sec, and ∆d is approximately 2.12 µm. To differentiate structures more finely in the z-axis, it is necessary to have a smaller ∆d. Obviously, lower focal plane speed results in a smaller ∆d, but more unexpected movement of the subject cornea will be included as the scan time is increased. Thus, there is a trade off between CMTF focal plane speed and CMTF sampling interval.

Using a property of NTSC video format, we found a way to halve ∆d by effectively doubling the frame rate r. In the NTSC standard, the video signal is in an interlaced format. A single video frame (1/30 second) is broken up into an odd field (1/60 second) and an even field (1/60 second), coming one after another in the camera output. The odd field is made up of the odd horizontal lines of the frame and the even field is made up of the even lines. By default, the image acquisition board digitizes the interlaced video signal and outputs the entire frame by combining the odd and even fields. However, this digitizing process is programmable. Normally CMTF images are acquired by subsampling every other pixel of the full video image in both the x and y directions, resulting in a frame 320 × 240 pixels in size (Fig. 4A). If the image acquisition board is programmed to sample all pixels in the y direction, but to subsample in the x direction, the digitized frame will have pixel data from every row and every other column of the full frame. By “de-interlacing” the rows of this 320 × 480 pixel frame, we obtain two half-size frames containing pixels from only the odd and even fields, respectively, and the CMTF image sampling rate is thus doubled (Fig. 4B). As expected, smooth transitions between the odd and even data points in the de-interlaced CMTF scans were observed, demonstrating the validity of the approach. Using this technique, high measurement
precision can theoretically be obtained even when using faster scan rates, which could minimize potential motion artifacts during a scan.

**Programming Techniques**

The new TSCM system required customized software to support its operation. The new program must integrate the following functions on line: (1) focal plane movement control; (2) image acquisition, processing, and display; and (3) intensity curve generation, calculation, and display. Microsoft Visual C++ (version 6.0) was used to develop the software, because it provides object-oriented design capability, which is ideally suited for development of multifunctional software. The object-oriented approach also eases the job of software maintenance and future functional enhancements. One of the challenges in developing this on-line scanning, on-line processing software was that several real-time processes must occur simultaneously. For example, the program needs to communicate with the Oriel 18011 lens motion controller continuously, even while acquiring images and performing 2-D and 3-D image processing and display. To address this issue, we used Microsoft Windows multithreaded programming and overlapped serial port communication techniques. Multithreaded programming allows several processes to run in parallel in optimized time slots, and overlapped serial port reading allows the program to perform other tasks while the serial port is waiting for a response from the lens controller. These advanced programming techniques optimize the use of computer resources and allow the program to be responsive enough for on-line operation.

**RESULTS**

Movement of the cornea due to pulse, respiration, or other factors often presents a significant challenge for in vivo confocal imaging. Although high-quality images can be obtained between eye movements, it is difficult to predict when a stationary sequence will occur. We have previously found that by acquiring 60 sequential frames that can subsequently be

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932909/)
reviewed, a quality stationary frame can almost always be identified (Fig. 5A). In many patients, several stationary images are observed between movements; to take advantage of this, we have incorporated a new temporal moving average procedure in TSCM2.0. As shown in Figures 5B and 5C, averaging of sequential images that are not aligned because of movement results in a blurred image. However, averaging over stationary periods provides an image with preserved edges and reduced noise compared with single frames (Fig. 5D). Typically, a 2- to 3-image-wide window can be used for the temporal moving average on human patients.

We have found that the most powerful and clinically useful new features in TSCM2.0 are on-line CMTF image acquisition and analysis and interactive 3-D display. Immediately after the completion of an on-line CMTF scan, the intensity curve, the side views, and the 3-D reconstructed volume projection with a default orientation and size are displayed. Because of the high computing power of the PC and the programming techniques used, updating these views occurs in real time (i.e., with no perceptible delay). Thus, the user can interactively pick a region of interest by dragging the corners of a highlighted "region of interest" box in the x-y plane, while the side views and the 3-D projection are continuously updated (Figs. 3, 6). This gives the user great power and flexibility for visualizing and localizing a structure inside the cornea interactively. The side views of the cornea have been particularly useful, because they provide a view similar to that of an ophthalmic slit-lamp (although at much higher resolution and contrast), which is familiar to most clinicians.

The 3-D reconstructions have been particularly useful for identifying and localizing the source of corneal haze after PRK (Fig. 6) and the flap interface after LASIK (not shown). CMTF has been used previously to estimate corneal haze quantitatively and objectively by calculating the area under the haze peak in the intensity profile. The previous CMTF program produced a numerical area value after the user typed in the start and end points of the haze peak using the keyboard. In this new software environment, area is calculated and displayed graphically by clicking on the peak start and end points (Fig. 6) with the cursor. Together with the interactive 3-D reconstruction and display, obtaining quantitative data on the amount and location of corneal haze becomes convenient and rapid.

**DISCUSSION**

Weigand et al. originally demonstrated using a scanning slit confocal microscope that by rapidly focusing through the cornea, a z-axis intensity profile can be generated. In their system, through-focus curves were generated by physically moving the entire objective lens to change the focal plane position within the cornea. One drawback to this technique is that the eye can move along the z-axis with respect to the lens tip during a scan, making it difficult to determine reliably the position of the focal plane within the cornea. Our system uses a specially designed objective lens, in which the tip of the lens is lightly appplanated to the cornea via a thin layer of methylcellulose, thus minimizing z-axis drift. The position of the focal plane relative to the objective tip is varied by moving the lenses within the objective casing (Tandem Scanning Corporation, Reston, VA). Using this lens, the position of the focal plane can be determined accurately. This is essential for obtaining accurate and reproducible measurements of corneal, epithelial, and stromal thickness. This specially designed lens is currently available from Advanced Scanning Limited (New Orleans, LA).

Patel et al. have previously reported using a Silicon Graphics Unix workstation-based system for acquiring CMTF...
images to the hard disk on line. However, to the best of our knowledge, ours is the first integrated TSCM system combining on-line scanning, processing, 3-D reconstruction, and analysis. By acquiring CMTF images directly to system RAM instead of the hard disk, real-time operation has been achieved on an inexpensive PC platform. Furthermore, once a 3-D dataset is saved to a file, all display and analysis procedures can be performed on any modern PC system (including lap-tops) without the need for any specialized hardware.

By sampling the odd and even video fields separately, we have also doubled the image sampling rate and theoretical measurement precision of CMTF. It is important to note the difference between “resolution” and “precision.” The resolution of the instrument is 9 μm, as defined by the full-width at half maximum of the through-focus response of the objective lens through a front surface mirror. This means that at least 50% of the signal from an object will be detected over a 9-μm depth. On the other hand, the precision of the CMTF measurements corresponds to the distance between adjacent frames (sampling interval). If we can identify the frame in which the object of interest is in the best focus, it is the precision that actually determines the lower limit (sensitivity) of our thickness measurements, as long as another interface or structure of interest is not present at the same x-y position within this 9-μm thick volume.

Of course, some limitations inherent in in vivo confocal imaging remain. First, the temporal moving average operation might become ineffective when in vivo eye movements relative to the objective tip become more frequent or more vigorous. Second, the 3-D reconstructed volume is somewhat distorted because of slightly unequal pixel dimensions in the x, y, and z directions; however, these renderings are clearly adequate for the visualization purpose for which they are intended. Finally, potential shifts in the x-y plane within the CMTF image stack due to eye movements have not been compensated for; thus motion artifacts are sometimes present in the 3-D reconstructions. Fortunately, occasional scans containing such movements can easily and quickly be identified.
and discarded with the new system. Image restoration techniques such as inverse filtering and blind deconvolution may be useful for removing motion blur.\textsuperscript{25,27} Misalignment of images in 3-D reconstructions could theoretically be minimized by performing a z-series image registration procedure before stacking the images together.\textsuperscript{18} The problem with these methods is that they are complex and do not operate in real time. However, they may be useful for subsequent off-line processing of saved 3-D datasets, in which processing time is not as much of an issue.

In conclusion, this is the first full-featured software package designed for in vivo TSCM imaging of the cornea that performs both 2-D and 3-D image acquisition, display, and processing as well as CMTF analyses. The convenience and performance of tandem scanning confocal microscopy have been greatly improved using this on-line scanning, on-line processing system. This advance should help to expand the use of in vivo confocal microscopy for both clinical and research applications. Readers interested in obtaining this new software should contact the corresponding author (WMP) via e-mail.

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References