Custom Plug-ins operating in UTHSCSA ImageTool Ver. 1.27

Computer-Assisted Microscopy and Digital Image Analysis
Software to Strengthen Microscopy-Based Approaches
for Understanding Microbial Ecology

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Direct any questions regarding CMEIAS not covered by this Dec. 2003 manual version or the Liu et al. 2001 Microbial Ecology publication cited above to Frank Dazzo at <cmeiasfbd@msu.edu>.
Microscopy is one of the most important techniques in microbial ecology, since this is the most direct approach to examine the microbe’s world from its own perspective and spatial scale. The value of quantitative microscopy in studies of microbial ecology can be increased even further when used in conjunction with computer-assisted image analysis. There are two main advantages of using digital image processing and pattern recognition techniques in conjunction with microscopy for quantitative studies of microbial ecology. First, automatic image analysis reduces the amount of tedious work with microscopes needed to accurately quantify *in situ* morphological diversity, abundance and metabolic activity of microbes. Secondly, these techniques provide an important quantitative tool that can significantly enhance the polyphasic analysis of the structure, diversity, spatial features, and functions of complex microbial communities *in situ* without cultivation.

One of the most important and yet most tedious tasks performed during microscopical analysis of microbial communities is the classification of observed cells into known morphological categories and recognition of new categories as well if new distinct characteristics are captured. Use of morphological diversity in evaluations of microbial community structure is more useful and valid if the cells are actively growing rather than in a non-growing state of quiescence, since the latter is more commonly associated with pleomorphic dwarf cells. This is because distinctive cell morphologies reflect the phenotypic expression of complex networks of genes involved in the synthesis and maturation of the shape-determining murein sacculus, plus other genes dedicated to the cell division cycle that are primarily expressed during active growth.

A major challenge in microbial ecology is to develop reliable and facile methods of computer-assisted microscopy that can analyze digital images of complex microbial communities at single cell resolution, and compute useful quantitative characteristics of their organization and structure without cultivation. Although several image analysis systems can classify microbes according to their cell size, automatic classification of cells according to their distinctive morphology (a dimensionless characteristic based on several shape features) represents a much more challenging task. Most commercial image analysis systems include some shape
measurement features that compute the roundness or circularity of cells, and these characteristics are sufficient to distinguish regular rods and cocci, the most common shapes of bacteria. However, the difficulty increases with morphological diversity, since automatic classification of most other microbial morphotypes requires measurement of multiple shape and size features to resolve the distribution of their morphological space. Some custom image analysis systems are adequate for automatic shape classification of spheres, straight rods, and vibroids or prolate spheroids. This represents the morphological diversity of some marine bacterioplankton communities. However, comprehensive image analysis systems capable of automatically classifying much broader morphological diversity in complex bacterial communities, as commonly exists in nutrient-enriched habitats containing actively growing bacteria that are larger in size and typically monomorphic, did not exist prior to development of CMEIAS®.

This recognition of the need to develop a comprehensive computer-aided image analysis system that could extract from images all the information needed to recognize and classify the morphological diversity component of microbial communities came to a pinnacle when the senior author was preparing photomicrographs of the microbial community in the bovine rumen for the cover illustration of the 9th Edition of Bergey’s Manual of Determinative Bacteriology (Fig. 1).

Fig. 1. **Actively** growing microbial communities contain a large diversity of bacterial morphotypes, as shown directly by this phase contrast light photomicrograph of bovine rumen fluid. Acquiring this image for the cover of Bergey's Manual was my spark of inspiration to develop CMEIAS.
That work clearly indicated the following key points:

- Contrary to current popular thinking about microbial community analysis, microscopy does reveal significant morphological diversity in complex, actively growing microbial communities.
- Automatic morphotype classification of complex communities exhibiting high morphological diversity will require development of a more flexible and robust computer-assisted image analysis system than those currently available.
- Phase-contrast light microscopy of dispersed samples immobilized on agarose-coated slides is a simple yet effective direct method to acquire images with the resolution and range of object brightness at high magnification that are sufficient to reveal the rich morphological diversity of actively growing microbial communities. Its essential requirements to detect microbes are that their size exceeds the \( \sim 0.2 \, \mu m \) limit for light microscopy, their refractive index differs from that of the surrounding medium, and high-quality optics are available to acquire the images.

We have improved on existing image analysis systems of computer-assisted microscopy by introducing new measurement features and robust object classifiers capable of automatically classifying most of the predominant microbial morphotypes encountered in digital micrographs of complex microbial communities growing in nutrient-enriched habitats, and have implemented these features in a flexible, user-friendly and robust semi-automatic image analysis system to strengthen microscopy-based methods for understanding microbial ecology. We named the program “CMEIAS\textsuperscript{©}”, an acronym for the Michigan State University Center for Microbial Ecology Image Analysis System. CMEIAS Ver. 1.27 is not a stand-alone program, but rather consists of several custom plug-ins designed to operate in the host program UTHSCSA ImageTool\textsuperscript{©} Ver. 1.27, a free downloadable open-architecture software operating on a personal computer. Two of these CMEIAS 1.27 plugins (objanal.dll and objclass.dll) are derived work, representing modified plugin versions of object analysis and object classification plugins and not the original UTHSCSA ImageTool distributed by the University of Texas. The accuracy of microbial morphotype classification has been thoroughly tested against ground truth data using CMEIAS v. 1.27 in UTHSCSA ImageTool running in Windows NT 4.0 (Liu et al. 2001). Similar results have been obtained using the Windows 2000 Professional operating system. Also, limited operational testing has indicated compatibility with Windows 95/ME/XP, but quantitative measurements of CMEIAS accuracy have not been evaluated for CMEIAS/ImageTool 1.27 using these alternative operating systems.

In summary, CMEIAS\textsuperscript{©} is an accurate, robust, flexible semi-automatic computing tool that fills a major gap by significantly strengthening microscopy-based quantitative approaches to understanding microbial ecology at spatial scales relevant to the microbe's niche, and should serve as a useful adjunct in the analysis of microbial community structure \textit{in situ} without cultivation.

Feel free to send feedback on CMEIAS so we can consider it in our upgrades currently under development. Direct questions not already covered by this operator manual or the Liu \textit{et al.} 2001 Microbial Ecology publication cited above to Frank Dazzo at <cmeiasfbd@msu.edu> ☺
Download and Installation of ImageTool / CMEIAS® Ver. 1.27 Image Analysis System

2.1 System Requirements

Hardware and Windows Operating System:
The minimum requirements to operate the host program UTHSCSA ImageTool ver. 1.27 include a PC with Intel 80486 running Windows NT 4.0 (Service Pack 4 or higher) / 2000 / ME / XP, 16 Mb RAM, and a monitor displaying 256 colors or higher with at least 800 x 600 pixel resolution. To operate CMEIAS v. 1.27 optimally within ImageTool, a PC with at least 233 MHz P-II and 64 MB RAM or higher are recommended. ImageTool can operate with various Windows-compatible printer and twain-compliant input devices. This operator manual is written with instructions for CMEIAS/ImageTool v. 1.27 operating in Windows NT 4.0 (SP6a), and so end-users must adjust instructions accordingly when using other operating systems. See Appendix V for known problems.

Essential Software: Uthscsa ImageTool Ver. 1.27 (host program into which CMEIAS v. 1.27 plug-ins are installed). Additional recommended: ImageTool can save data directly or copy them to the Window’s clipboard so they can be pasted into a compatible spreadsheet program (e.g., Microsoft Excel®). Adobe Acrobat Reader is needed to read/print this operator manual and the tutorial worksheet. Additional software to edit images (e.g., Adobe Photoshop®, Image Processing Tool Kit®) and perform ecological statistics (e.g., Trinity EcoStat®) are recommended.

2.2 Download and installation of ImageTool/ CMEIAS® Ver 1.27

CMEIAS Ver. 1.27 image analysis software is not a stand-alone program application. Its various files must be installed manually into specific folders of UTHSCSA ImageTool Ver. 1.27 and operate in this host program. CMEIAS Ver. 1.27 will not operate in ImageTool Ver. 2.x or higher. An upgrade
of CMEIAS for UTHSCSA ImageTool Ver. 3.0 is under development. Complete and submit our online “Join Our CMEIAS Mail List” page at the CMEIAS website for updated information.

1. Open NT Explorer. Select View > Options > Show all files to display the dll plug-in file names. Users may need administrator privileges to do this step.
2. Create a folder target location called IT-CMEIAS download on your computer to temporarily store the downloaded ImageTool and CMEIAS files before installing them.
3. Open the UTHSCSA ImageTool web site at http://www.ddsdx.uthscsa.edu/dig/itdesc.html
4. Read the contents of the ImageTool web site. Locate the Overview and then click Download Software under Overview.
5. Scroll down to the ImageTool Application Version 1.27 heading (not Ver. 2.x or 3.0).
6. Right click on Disk 1, choose Save target as… and save the file (it127d1.zip) to your newly created IT-CMEIAS download folder. Do the same procedure for disks 2 & 3.
7. Unzip the it127d1.zip, it127d2.zip and it127d3.zip files in your IT-CMEIAS download folder.
8. Double-click the Windows Wizard Setup.exe file to install IT Ver 1.27 onto your C drive (C:\Program files\). This installation creates the Uthscsa main directory folder and ImageTool subfolder under C:\Program files. (Fig. 2).
9. Open the CMEIAS website (http://cme.msu.edu/cmeias/) and read its contents, including the License Agreement. Downloading CMEIAS files constitutes your acceptance of the terms of this License Agreement. The CMEIAS and UTHSCSA ImageTool license agreements are also copied in the beginning of this operator manual.
10. For download purposes, all CMEIAS 1.27 program and tutorial files are combined into a zip file called Cmeias127.zip. At the CMEIAS website Download Area page, download the Cmeias127.zip file into the IT-CMEIAS download folder in your computer (#1A of Fig. 2).
11. Unzip this downloaded Cmeias127.zip file containing the following 4 folders and 32 files:
   a. The CmeiasIcon.ico file contains the optional CMEIAS Icon to replace the ImageTool icon shortcut on your desktop (#1A of Fig. 2).
   b. Installation of CMEIAS v. 1.27.txt is a "ReadMeFirst" text file of these instructions (#1A of Fig. 2).
   c. The Cmeias folder contains the dict subfolder with 16 morphotype classification label files (#1A of Fig. 2).
   d. The CMEIASplugins folder contains the objanal.dll, objclass.dll, and objlabel.dll files (#1B of Fig. 2).
   e. The CmeiasHelp folder contains all the files for the CMEIAS 1.27 training tutorial and recorded macros (#1C of Fig. 2), including:
      • the interactive training tutorial macro (Cmeias127trainingTutorial.itm),
      • 3 recorded macros for object analysis and classification (CmeiasIT-1objectClassification.itm, CmeiasMorphotypeClassification.itm, CmeiasObjectAnalysis.itm).
      • the training tutorial data worksheet (CmeiasTutorialWorksheet.pdf),
      • 3 segmented tiff images of bacteria (CoccusRegularRodFilament.tif, Community-A.tif and Community-B.tif),
      • 2 calibration files for the IT/ CMEIAS-1 classifier (OptimizedAreaBins.ocd and OptimizedWidthLengthBins.ocd),
      • instructions for installing and running the CMEIAS training tutorial and recorded macros (ReadMeFirstCmeiasMacros.txt).
12. Download the Cmeias127.pdf file of this CMEIAS v. 1.27 operator manual from the CMEIAS website (#2 of Fig. 2).
Perform the following steps to install CMEIAS v. 1.27 into Uthscsa ImageTool v. 1.27:

1. Copy the Cmeias folder and all its contents into the Uthscsa\ImageTool folder (# 3).

2. Copy the 2 ocd files from the CmeiasHelp folder into the Uthscsa\ImageTool\Calibration folder (# 4A).

3. Copy the CmeiasTutorialWorksheet.pdf, CoccusRegularRodFilament.tif, Community-A.tif, Community-B.tif, ReadMeFirstCmeiasMacros.txt files (all in the CmeiasHelp folder) and this Cmeias127.pdf operator manual file into the Uthscsa\ImageTool\Help folder (# 4B).

4. Copy the 4 itm macro files from the CmeiasHelp folder into the Uthscsa\ImageTool\Macros folder (# 4C).
5. Open the ImageTool Plug-Ins folder to display its contents (all of #5 in Fig. 2). Remove the ImageTool objanal.dll and objclass.dll plugin files (#5A of Fig. 2) and paste them elsewhere in a secure place. Then copy the 3 CMEIAS dll plugin files from the CmeiasPlugins folder (#1B) into the ImageTool Plug-Ins folder (#5B of Fig. 2). Note: If at a later time you wish to run the original ImageTool plugins, simply remove the corresponding CMEIAS 1.27 files before reinstalling them.

6. (optional) Copy the CmeiasIcon.ico file into the C:\Program Files\Uthscsa\ImageTool folder and follow instructions in section 2.3 for it to replace the icon of the ImageTool shortcut on your desktop.

Note: If your computer already has UTHSCSA ImageTool ver. 3.0 installed in a C:\Program Files\Uthscsa\ImageTool folder, then you can still install (and run CMEIAS v. 1.27 in) ImageTool v1.27, but during the setup.exe wizard installation of ImageTool, you must specify a different folder name (e.g., C:\Program Files\Uthscsa\ImageTool-1.27) as the destination of the ImageTool 1.27 program files. In this case, you must also assign a different name for the desktop icon so it won’t conflict with the ImageTool v3.0 desktop icon. Do not install CMEIAS v. 1.27 plugins in ImageTool v. 3.0.
2.3 Installation of our CMEIAS desktop shortcut icon to launch the program

The installation of UTHSCSA ImageTool 1.27 automatically places a desktop “IT 1.27” shortcut icon targeted to open the “it.exe” file (located in C:\program files\Uthscsa\ImageTool). We have included an optional CMEIAS-IT icon file CmeiasIcon.ico in the downloaded Cmeias127.zip that can replace the ImageTool desktop shortcut icon to launch this program (Fig. 2 item # 6 and Fig. 3).

![CMEIAS-IT Icon](image)

**Fig. 3.** This snazzy desktop icon illustrates 7 of the microbial morphotypes classified by CMEIAS (coccus, regular rod, branched filament, spiral, ellipsoid, U-shaped rod, and club) displayed according to their assigned pseudocolors. Install it in the ImageTool root directory as indicated.

(Optional) Replace the IT icon on your desktop with this CMEIAS-IT shortcut icon as follows:

1. Open the downloaded **Cmeias127.zip file** and copy the **CmeiasIcon.ico file** into the Uthscsa\ImageTool folder (Fig. 3).
2. Right-click the **it.exe** shortcut icon previously installed on your desktop.
3. Select **Properties > Shortcut tab**, then select **“Change Icon”**.
4. Click **Browse**, go to the C:\ Program Files\Uthscsa\ImageTool folder.
5. Left-click once the **CmeiasIcon.ico file**.
6. Click **Open**; the CMEIAS icon image should now appear in the current icon white bar.
7. To properly display all colors in the CMEIAS shortcut icon (e.g., spiral bacterium should be orange in color), you may have to make an adjustment in the **Display control panel** of your computer. If so, open the Plus! Tab (Start > Control Panel > Display > Plus!), click the box to **“Show icons using all possible colors”**, then click **Apply and Close OK**.
8. If you are instructed to restart your computer to activate this shortcut icon change, do so at this time. The pseudocolors for the various microbial morphotypes in the CMEIAS-IT shortcut icon should now display properly on your desktop.
9. To start the CMEIAS-ImageTool 1.27 program, double-click this shortcut icon on your desktop, or click on the Windows **Start** button, and then select Programs > UTHSCSA ImageTool > ImageTool.
10. A quick way to check that the CMEIAS 1.27 plugins have been installed properly in ImageTool is to verify that the unique CMEIAS options for object analysis and object classification display in the corresponding tab pages (Main Menu Settings > Preferences > Measurement Features, or Preferences > Object Classification) as illustrated in Figs. 6 and 25, respectively.
3 Microbial sample preparation, microscopy and image preparation

Semi-automatic image analysis of microbes can be principally divided into five stages:
1. image acquisition and digitization
2. interactive image editing and segmentation to locate the foreground cells of interest
3. automatic extraction of selected measurement features for each object found (object analysis)
4. classification of different cell types (object classification)
5. computations, statistical analysis, and interpretation of data.

3.1 Essential steps required before accurate morphotype classification of microorganisms using CMEIAS image analysis

1. The first requirement for accurate image analysis is to produce a very high quality primary image using any type of microscopy (e.g., brightfield LM, phase contrast LM, TEM, SEM, CLSM) that can distinguish the contour of each foreground microbial cell of interest from the background. Low quality images are the most frequent cause of unexpected, inaccurate and unreliable results using CMEIAS. For CMEIAS image analysis, the primary image can be acquired in photo, video, or digital format, but it must be converted to an 8-bit digital grayscale image in an uncompressed file format [e.g., Tiff, BMP] in order to be analyzed and classified in Uthscsa ImageTool/CMEIAS Ver. 1.27. RGB color images can be opened and analyzed manually (but not automatically) in ImageTool (see section 5.8 “Manual Object Counting”).

2. Digital images must have adequate magnification and pixel resolution so that even the smallest cell of interest has sufficient pixel sampling density [at least 30 pixels] to define its contour for accurate morphotype classification. This requirement poses no problem for skilled microscopists using research-quality optics and a modern computer.
3. Prior to analysis, the image must be edited sufficiently so that it can be reduced to the foreground objects of interest using the brightness thresholding procedure in ImageTool. This image editing must precede CMEIAS morphotype classification. ImageTool Ver. 1.27 features some image editing routines, but digital images of microbial communities will often require additional interactive editing using other image processing programs to achieve full segmentation, e.g., splitting of touching objects, removal of invalid objects or background pixels whose brightness values lie within the range that defines the foreground objects of interest. Consult John Russ’s excellent Image Processing Handbook (2002, 4th ed.) and his Image Processing Tool Kit for details of the theory and practice of digital image segmentation.

4. Before performing object analysis and object classification in ImageTool / CMEIAS, the user must select various setting preferences (Settings > Preferences > …) on tab pages labeled Find Objects, Image, Measurement Features and Object Classification, then open and spatially calibrate the image, and find the objects of interest by the brightness threshold segmentation step(s). After extraction and computation of selected measurement attributes from each foreground object, the quantitative image analysis data reported in the ImageTool “Results Window” spreadsheet are ready for copy/paste export to an external windows spreadsheet program where they are further processed and analyzed statistically.

We include here some recommendations to produce good images for quantitative image analysis, starting with the sample preparation to immobilize bacteria on agarose-coated slides. The goal is to produce phase-contrast micrographs containing the refractile bacteria immobilized in the same flat focal plane on the agarose surface, and at an ideal recommended spatial density of ca. 30-170 bacterial cells per field of view. If necessary, dilute (or concentrate) the cell suspension using filter-sterilized water or culture medium and prepare additional slides to produce this ideal spatial density of bacteria. Cell densities lower than 30 will require too many images to produce a statistically adequate sample size for the data set, and densities higher than the maximum recommended 170 will likely contain many touching cells that must be separated by image editing procedures prior to analysis, plus cells often too small (< 30 pixels) for morphotype classification.

3.2 Preparation of the agarose-coated slide

1. Wash a high quality agarose (e.g., Boehringer Mannheim LE agarose; Cat. No 1685651) four times with deionized water (aspirate supernatant fluid following sedimentation).
2. Prepare a 1.6% (w/v) suspension of the washed agarose in deionized water in screw cap bottles and autoclave them (121 °C for 15 min).
3. Clean several glass microscope slides with frosted ends and wipe dry with clean tissue paper.
4. Place slides horizontally and spaced apart on a perfectly leveled surface that is checked with a bubble spirit level. (Note: lab benchtops may have areas that are not perfectly level).
5. Dispense 1.6 ml of the dissolved tempered agarose solution from a pipette in a zigzag motion over the clean smooth surface of each slide (frosted end side up) without allowing overflow.
6. Cover (without touching) the slides with a large inverted glass dish until the agarose solidifies.
7. Dry the agarose-coated slides overnight in a horizontal position at 50 °C in a desiccator oven.
8. Store the dried agarose-coated slides in a clean slide box until used.
3.3 Preparation of the dispersed microbial sample

1. Label the frosted end of the slide with the sample name(s).
2. Pass the suspended sample of the microbial community rapidly through a 25-gauge needle several times to assist in achieving a uniformity of single cell dispersion.
3. Dilute the dispersed sample until it has a barely visible slight turbidity (~10^7 cells/ml).
4. Immediately before microscopy, deposit exactly 26 µl of the sample to a confined area of the dried agarose-coated slide. This volume is optimized for the next step; adjust if using a different coverslip size. Each slide can accommodate two samples.
5. Carefully apply a cleaned 22 x 22 mm coverslip to the suspended sample without trapping air bubbles. The coverslips should have the proper thickness matched to your 100X phase contrast objective lens (commonly # 1½ which is 0.17 mm thick).
6. Vary the sample volume as needed to completely fill the volume beneath the coverslip with the edges remaining dry. Any excess sample on the slide outside the coverslip should be wicked into a forceps-held small square of filter paper. The fluid volume of the sample under the coverslip will be absorbed within a few minutes by the rehydrating agarose gel layer with no free fluid remaining. Prepare only one slide at a time and store it horizontally in a portable humidity chamber until examined microscopically.

3.4 Phase-contrast microscopy of refractile, immobilized cells

1. Because image analysis requires a high quality primary image, strict adherence to the principles of Köhler illumination with proper phase condenser alignment and uniform background illumination is essential. Consult your microscope operator manual for details.
2. (Optional, recommend) Introduce a narrow band-pass green (546 nm) interference-contrast filter (e.g., Omega Optical #XF1020) beneath the phase-contrast condenser to increase resolution by reducing chromatic aberration. The increased contrast and improved quality of the grayscale image resulting from use of this filter is well worth its cost. Also, digital images with transmitted illumination acquired by a CCD camera may require removal of a central bright spot by introducing an infrared-absorbing filter in the light path.
3. For morphotype classification analysis, use a flat-field corrected, 100X phase 3 oil immersion objective (e.g., PlanApochromat).
4. The numerical aperture (N.A.) of the oil immersion system is a function of the N.A. of the objective and the condenser. Since the N.A. of this oil immersion objective is >1, the condenser beneath the slide should also be oiled to fill the gap between the condenser and the slide in order to realize their full N.A. If the condenser is not oiled, the highest possible N.A. is 1, regardless of the N.A. of the objective.
5. Find fields of view in which the refractile bacteria are optimally separated from one another. Due to edge drying, cells close to the edge of the coverslip are often immobilized first. Ideal fields to photograph are ones that contain very refractile cells that HAVE JUST BEEN immobilized. Bacterial cells will eventually lose some refractility as the agarose continues to swell further and completely surrounds them.
6. Acquire micrographs from as many different locations as are feasible so that a sufficient number of randomly selected cells are sampled for community analysis.

- **Note**: The number of images required to capture the entire morphological diversity of the community examined will depend on its morphotype richness and distribution of abundance of morphotypes present, and the spatial density (hence total number) of the cells within the images. The sample size is adequate when a plot of cumulative morphotype diversity index vs. cumulative sample size rises to a plateau.

### 3.5 Image acquisition and digital image requirements

CMEIAS/ImageTool 1.27 performs automatic object analysis and morphotype classification on **8-bit grayscale images without compression**, acquired either directly via Twain-compliant devices (digital camera, film and/or flatbed scanner, “frame-grabber” image capture board for live or pre-recorded video image; see corresponding operator manuals for details), or previously acquired digital images saved in one of ImageTool’s 22+ PC-compatible image file formats (Tiff, BMP, etc). ImageTool will not work with LZP compressed Tiff images.

The measurement precision and accuracy for object analysis and morphotype classification strongly depend on the **sampling density** of the image, defined by its pixel resolution (pixels per unit image length). A disadvantage of digital microscopy as compared to photomicrography is that digitization samples objects at higher error rates. This is because pixels are typically larger and have square corners in contrast to the smaller, more rounded silver grains in photographic film. The digital image can have a significantly lower sampling density, hence more jagged, less accurately defined object contours. Acquisition of microbial images at insufficient pixel resolution is indicated if the jagged contours of regular rod-shaped (blue pseudocolored) bacteria cause CMEIAS to misclassify them as (yellow pseudocolored) prosthecates. This source of error can be avoided by acquiring images at high resolution. We use 1200 dpi for 35mm film scan, 1000 horizontal lines per inch for video frame-grabber, and maximum native resolution of digital cameras followed by bicubic interpolation in Adobe Photoshop to mathematically increase resolution (see next section 3.6). This large sampling density creates large image files that require large file storage capacity and computer RAM to save and analyze them, respectively. Images must have adequate magnification and resolution so that even the smallest cell of interest has sufficient pixel density [at least 30 pixels] to define its contour for accurate morphotype classification. This requirement poses no problem for skilled microscopists using research-quality optics and a modern computer. To facilitate image archiving, we typically save the original grayscale image in Tiff format (always keep a backup copy elsewhere!), and the corresponding edited images in BMP format. Several image archiving softwares are available to help manage large image data sets.

### 3.6 Adjustments in size and pixel resolution of images acquired using a digital camera

When an image acquired using a high resolution digital camera is opened in Adobe Photoshop (ver. 5 or higher), the user can check its dimensions by selecting **Image Size** under the
Image menu. Fig. 4A is a screen shot of the Image Size entries for a Digital Instrument’s Spot image as it comes into Photoshop. The next step is crucial in order to adjust the image size to one that would be typically displayed at 1:1 and analyzed in CMEIAS/ImageTool without creating problems in pixel resolution. By default, the Resample Image box is checked in the Image Size dialog box (Fig. 4A with an arrow and the Bicubic resampling algorithm selected). Photoshop arbitrarily assigns 72 pixels/inch resolution to all unfamiliar images, and then automatically multiplies the image’s pixel width and length (here: 1315 x 1033 pixels) by the default 72 pixels/inch to report a print size of 18.264 inches x 14.347 inches. If a smaller Print Size dimension (e.g., 5 inches width) is entered while the Resample Image box remains checked, Photoshop will reduce the Pixel Dimensions by the factor required to maintain pixel resolution at 72 pixels/inch. This greatly reduces the pixel dimensions of the image, resulting in a poorer quality image that cannot be analyzed optimally. The solution is to uncheck the Resample Image box (Fig. 4AB arrow) before reducing the image size. This setting maintains the pixel dimensions of the original image. For this example illustrated in Fig. 4B, the image is resized to 5 inches wide (automatically the height becomes proportionally resized to 3.028 inches) and this automatically increases the pixel resolution to 263 pixels/inch to maintain the 1315 x 1033 Pixel Dimensions of the original image at the new, reduced print size. An image resized this way looks great and can be analyzed accurately using CMEIAS/ImageTool. For more information on this and related topics relevant to image acquisition and digital image adjustments, see the “Frequently Asked Questions” page in the Digital Instruments website at: <http://www.diaginc.com/faq.htm#k>.

3.7 Preliminary Image Editing

Before performing image analysis, the pixels that define the foreground objects of interest must be distinguished from those of background. The various image processing procedures used to prepare an image so that the foreground objects of interest can be isolated by computer vision is collectively called segmentation. Objects in images to be analyzed by CMEIAS / ImageTool are
ultimately found by a brightness thresholding procedure, which requires that all the pixels that define the foreground objects of interest must have brightness values outside the range of image background. In some cases, the quality of the grayscale image (i.e., its signal-to-noise ratio) allows it to be segmented directly in ImageTool by the thresholding procedure alone (illustrated later in Fig. 20). ImageTool provides several image processing routines (contrast manipulation, sharpening, smoothing, median filtering, dilate/erode, spatial convolution with user-defined convolution inputs, background subtraction, interactive histogram, image stack averaging, etc.) to further edit the image before threshold segmentation. Consult the UTHSCSA Image Tool operator manual for pertinent details. More commonly however, images of microbial communities contain pixels of invalid objects or other background noise whose brightness levels fall within the range that defines the foreground objects of interest, or the bacteria are touching one another thus requiring other image editing steps to achieve segmentation prior to analysis by CMEIAS / ImageTool.

Examples of interactive editing steps to prepare images for threshold segmentation include:

- Increase the contrast of selected objects, e.g., thin filament, spirals
- Split touching objects with a continuous white line that is thresholded as background
- Add some noise-free margin to the image so foreground objects of interest can be fully included
- Optimally adjust the histogram of grayscale intensity levels or curves
- Fill "holes" in selected foreground objects with black pixels
- Remove invalid objects whose pixel brightness is in the range of foreground objects of interest
- Apply a median filter to smooth object contours without changing their overall shape or size

The ideal combination of image editing procedures will vary, depending on the types of segmentation problems encountered (e.g., invalid objects, touching objects, grayscale background pixels within the brightness range for the objects of interest). More details on the editing procedures used to segment images of bacteria are presented in Liu et al. 2001 Microbial Ecology 41: 173-194 and John Russ's Image Processing Handbook (CRC Press). Some image processing plugins (e.g., J. Russ, Image Processing Tool Kit®) can be installed in Adobe Photoshop® or Uthscsa ImageTool to edit images so the objects of interest can be found properly by the threshold segmentation routine.

3.8 Adding a calibrated magnification bar scale to the digital image

Images intended only for shape analysis / morphotype classification need no spatial calibration (default unit is the pixel) since shape measurements are unitless. On the other hand, all measurements of cell size require that the image is spatially calibrated in order to convert the default dimension of pixels into units of the measurement feature used, no matter what zoom factor is chosen for the image analysis. This can be done in ImageTool by either of two ways: (1) spatially calibrate at high zoom using a magnification bar scale of known length embedded directly in the image, or (2) load a spatial calibration file saved from a previous image of a slide stage micrometer acquired at the same magnification and pixel resolution (Settings > Load Spatial Calibration > (select appropriate “ite” file) > Open. The use of an appropriate spatial calibration file is recommended when analyzing many images at the same magnification, since it eliminates the need to add a bar scale to each image.
Fig. 5 shows the use of an image editor program (e.g., Adobe Photoshop) to create a black rectangular bar scale of 10 $\mu$m directly on a digital micrograph of a micrometer taken at the same magnification and pixel resolution as the image to be analyzed, and then copy / paste it onto an uncrowded corner of the segmented image. Note that the ends of the bar scale line are made exactly at the same relative position (left edge) of the slide micrometer vertical lines separated by 10 $\mu$m.

Some digital camera operating systems can automatically add a bar scale directly on the digital image. Also, a free, downloadable “Enter Magnification” plug-in (48KB zip file) that automatically puts scale bars on micrographs in Adobe Photoshop (version 4 and higher) is available from John Russ, North Carolina State University at http://www.reindeergraphics.com/free.shtml. Unzip and install it into the plug-ins folder of Adobe Photoshop. This plugin requires user-input information on the image magnification, the dots per inch (dpi) in which the image was acquired, and the length of the bar desired in micrometers. The program draws and labels the bar scale in the lower right corner of the image using the selected foreground and background colors.

Fig. 5 shows a phase-contrast grayscale micrograph of a methanogenic bioreactor community (left), the corresponding edited image with segmented foreground objects (right), and the micrometer image at the same magnification (bottom) used to produce the 10 $\mu$m bar scale (in Adobe Photoshop) that is pasted into the binary image of bacteria for spatial calibration.
Setting ImageTool and CMEIAS preferences prior to image analysis

ImageTool features many setting preferences (Settings>Preferences>various tab pages) to address before performing an image analysis. Several ImageTool default settings should be retained for CMEIAS operations whereas others need to be changed. The following preference settings for Measurement Features, Find Objects, and Image pages are relevant to image analysis of bacteria using CMEIAS. Consult the ImageTool Ver. 1.27 Operator Manual (C:\UTHSCSA\ImageTool\Help folder\it.doc) for other preference settings not indicated here.

4.1 Measurement features used in object analysis

4.1.1 Graphical interface for measurement feature selection

Fig. 6 shows the Measurement Features page (Settings > Preferences > Measurement Features) to select which attributes are to be extracted from each foreground object found within the image. Any combination of measurement features can be selected. The Object Analysis plugin in UTHSCSA ImageTool v. 1.27 contains 19 measurement features, including object area, perimeter, Feret diameter, major and minor axis lengths, roundness, elongation, compactness, major and minor axis angle, gray centroid, integrated density, min / mean / median / mode / max gray level densities and their standard deviation, and centroid [x, y] coordinates. New measurement features of CMEIAS Ver. 1.27 added to this Object Analysis plug-in include maximum curvature, length, width, width/length ratio, length/width ratio, area/bounding box area ratio, eight Fourier descriptors, and aspect ratio.
Fig. 6. Interface selection page (Settings> Preferences>Measurement Features) to activate the various measurement features of CMEIAS / ImageTool. The specific set of 7 features (actually 14; eight Fourier Descriptors are grouped together as one selection) enclosed within the gray line-framed box on the left represent those required to operate the CMEIAS-2 Morphotype Classifier. Features selected on this page are displayed as column headings in the object analysis Results window and the corresponding data array for each object found within the image analyzed.

### 4.1.2 Definitions of measurement features for object analysis

The measurement features important in the image analysis of microbial morphotypes are:

**Area**: Area of the object, measured as the number of pixels (scaled to the user-defined unit for image calibration) in the polygonal approximation of the cell. This measurement of size tends to slightly over-estimate the object's true area because the borders of the pixels may extend beyond the true perimeter of the cell.

**Perimeter**: Length of the outside contour of the object represented as a polygon.

**Roundness** (also called circularity shape factor): Computed as \(4 \pi \frac{Area}{Perimeter^2}\). This shape feature measures the degree of object roundness. Values lie between 0 and 1. The greater the value, the rounder is the object.

**Major Axis Length**: Length of the longest line that can be drawn through the object, corresponding to the vector \(CD\) in Figure 7a.
**Minor Axis Length**: The length of the longest line that can be drawn through the object perpendicular to the major axis. This size measurement is the vector $EF$ in Figure 7a.

**Elongation**: The ratio of the length of the major axis to the length of the minor axis, i.e., $CD/EF$ in Figure 7a. The result is a value $\geq 1$. If the elongation is 1, the object is roughly circular or square. The ratio increases from 1 as the object becomes more elongated.

**Compactness**: Computed as $\left(\sqrt{4 \text{Area}/\pi}/\text{Major Axis Length}\right)$. This shape feature measures the object's circularity, representing the ratio of the Feret diameter to the object's major axis length, and ranges between 0 and 1. Objects with a compactness value of 1 are roughly circular.

**Maximum Curvature**: The curvature at a point on the boundary of an object is defined as the inverse of the angle at that point; hence the maximum curvature has the minimum angle on the object boundary. The angle itself is defined as the angle between two equidistant strings (each set at a length of eight pixels) emanating from the point. In Figure 7a, the angle at point $D$ is $\angle GDH$, where $GD = HD$. To compute the local angle, the polygonal representation of the boundary is resampled at a constant interval along the object boundary.

**Length, Width, Width/Length Ratio, Length/Width Ratio**: The length of the object should be theoretically computed along its principal skeleton, which are the loci of centers of maximal disks.
contained in the object. However, in terms of accuracy and computational cost, it is not easy to
extract a useful skeleton since it is very sensitive to boundary noise. The closest approximation to
cell length provided by ImageTool is the major axis length (defined above, also called the “longest
dimension”). Because this measurement feature can significantly underestimate the length of curved
cells (line CD in Fig. 7a), we adopted an alternative “adaptive” algorithm to measure cell length
automatically in CMEIAS. This algorithm first classifies the objects according to their roundness
value, and then applies the appropriate formulas to compute cell lengths and widths for each
roundness class. In the first step, objects are automatically classified into one of two types:

i) **elongated** if $\text{Roundness} \leq 0.8$, and

ii) **rounded** if $\text{Roundness} > 0.8$.

Referring to Fig. 7b, a 2-dimensional presentation of a straight rod with rounded ends can be
represented by a rectangle attached to a half-circle at each of its two poles, and its length can be
approximated as $(a+b)$. On the other hand, we use the *Major Axis Length* feature to define the length
of a more rounded object. Thus, the CMEIAS formula for the object length is as follows:

$$
\text{Length} = \begin{cases} 
\frac{2\text{Perimeter} + (\pi - 2)\sqrt{\text{Perimeter}^2 - 4\pi\text{Area}}}{2\pi}, & \text{if Roundness} \leq 0.8; \\
\text{Major Axis Length}, & \text{otherwise}.
\end{cases}
$$

The algorithms to measure cell length and the major axis length return values that are equal in
accuracy for elongated microbes with a straight longitudinal axis, e.g., regular rod, but the algorithm
in CMEIAS used for cell length is more accurate than the major axis length for microbes with a
curved axis, e.g., curved rods, U-shaped rods, spirals, bent unbranched filaments (CD in Fig. 7A
illustrates the problem), and represents a significant strength of CMEIAS object analysis. ☺

The width of an object is defined as its average width along the skeleton and is approximately
computed for these two types of objects as follows:

$$
\text{Width} = \begin{cases} 
\frac{\text{Perimeter} - \sqrt{\text{Perimeter}^2 - 4\pi\text{Area}}}{\pi}, & \text{if Roundness} \leq 0.8; \\
\text{Area} / \text{Major Axis Length}, & \text{otherwise}.
\end{cases}
$$

The ratios $\text{Width}/\text{Length}$ and $\text{Length}/\text{Width}$ between the measurements $\text{Width}$ and $\text{Length}$ calculated
using the above formulas are dimensionless normalized measures of cell shape.

**Area/Bounding Box Area**: This unitless shape measurement feature is the ratio between the object’s
area and the area of the smallest rectangle enclosing the object. The four boundaries of the minimum
enclosing rectangle are parallel to the major axis and minor axis, respectively. This measurement of
shape is approximately computed as $(\text{Area}/(\text{Major Axis Length} \times \text{Minor Axis Length}))$. 

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Fourier Descriptors: Fourier descriptors are shape measurement features derived from the object contour and can be used to represent open or closed curves at different spatial scales. In addition, shape features can be extracted from Fourier descriptors which are invariant to translation, scaling and rotation. To compute the Fourier descriptors, the object boundary, represented as a polygon, is resampled by a sequence of equidistant points \((x_k, y_k)\), \(k = 0, ..., N - 1\), where the distance between the neighboring points is a constant. Let \(z_k = x_k + jy_k\), \(k = 0, ..., N - 1\) be a sequence in the complex space. Then \(z_k\) can be represented by its discrete Fourier transform coefficients

\[
z_k = \sum_{n=0}^{N-1} a_n e^{\frac{jn2\pi}{N}}, \quad k = 0, ..., N - 1,
\]

where

\[
a_n = \frac{1}{N} \sum_{k=0}^{N-1} z_k e^{-\frac{jm2\pi}{N}}, \quad n = 0, ..., N - 1
\]

are the discrete Fourier transform coefficients and \(a_0\) is the mean of \(z_k\), \(k = 0, ..., N - 1\).

Let

\[
z'_k = Sz_k e^{j\varphi} + T, \quad k = 0, ..., N - 1
\]

be a distortion of \(z_k\), where \(S\) is the scaling coefficient, \(T\) the translation vector in the complex space, \(\varphi\) the rotation angle and \(t\) the deviation of the starting point. The corresponding Fourier coefficients are:

\[
a'_0 = a_0 + T,
\]

\[
a'_n = a_n S e^{-\frac{jnm2\pi}{N}} e^{j\varphi}, \quad n = 1, ..., N - 1.
\]

If \(t\) is not an integer number, this equation is an approximate equality and the degree of approximation is dependent on the difference between \(t\) and its nearest integer number. It can be proved using the property of this equation that features \(f_n = |a_n|/|a_1|, n = 2, ..., N - 1\) are invariant with respect to translation, scaling and rotation. Since low-order Fourier coefficients occupy most of the energy of the signal, we use \(f_2, f_3, f_4, f_5, f_{N-1}, f_{N-2}, f_{N-3}\) and \(f_{N-4}\) as 8 Fourier descriptor (FD) features in CMEIAS®. When the Fourier Descriptors feature is selected in the Preferences > Measurement Feature page (Fig. 6), 8 columns for all 8 Fourier descriptors will be labeled FD0~FD7 in the object analysis Results window (Fig. 8). Heavy CMEIAS math ☺.

Feret Diameter: Diameter of a circle having the same area as the object, computed as \(\sqrt{\frac{4 \text{Area}}{\pi}}\).

Centroid \((x, y)\): The center point \([x, y]\) intercept (labeled \(O\) in Fig. 7a) is computed as the average of the \(x, y\) coordinates of all pixels belonging to the object. This attribute reports the Cartesian \(x, y\) coordinates that locate every object found in the image relative to the landmark origin selected in the Settings>Preferences>Image preference page (see section 4.3.1). For ImageTool v. 1.27, these object centroid values are in pixels regardless of the unit of length used to calibrate the image. (They are reported in the user-defined units in the upgraded ver. 3.0). Note: the centroid pixel (mean of all object pixel \(x, y\) coordinates) may locate outside the contour of curved or irregularly shaped cells.

Aspect Ratio: This shape measurement attribute is the ratio between the minimum and the maximum distance from the points on the object’s boundary to its centroid, \((OB/OA\) in Fig. 7a). Note: a special situation arises with the geometry of some curved cells: the aspect ratio algorithm will correctly compute a value of 0 when the centroid lies at the cell contour.
Major Axis Angle, Minor Axis Angle, Gray Centroid x/y, Integrated Density, Min. Mean, Median, Mode, Max. and Std. Dev. Gray Level are measurement features provided by ImageTool that are not used by CMEIAS to classify bacterial morphotype at this time. See the ImageTool Operator Manual (It.doc) for information on these other measurement features.

4.1.3 Measurement Precision (decimal places)

Enter this number in the Settings > Preferences > Measurement Feature page (Fig. 6) to set the decimal places for the data output of the object analysis Results window. Since images of bacteria are typically calibrated in micrometers (µm) and the resolution limit for light microscopy is ∼0.2 µm, the precision should be set at 1 or 2 decimal places for linear or area measurements, respectively. Use 2 if both are measured.

4.1.4 Display of object analysis data in the Results Window

Following object analysis, each selected measurement feature appears as column headings in the ImageTool Results window spreadsheet (Fig. 8), and the corresponding values extracted from each foreground object found in the image are reported individually in rows in units that are designated during the “calibrate spatial measurement” step (also see section 5.6). The mean and standard deviation for all measured values in each column array are automatically computed and displayed in gray filled cells of the first 2 rows above the first object row of data (Fig. 8). These reported statistics are useful for object analysis but not for morphotype classification.

The only exceptions to this display design is with the Centroid x, y and Gray Centroid x, y data, which each display as 3 columns: one column of ImageTool design contains both the x and y coordinate values delimited by a comma, and two columns of CMEIAS design list the centroid X and Y coordinates separately (Fig. 8). This latter CMEIAS display design facilitates the export of these spatial coordinates into other software applications for geostatistical analysis of object spatial distribution.

<table>
<thead>
<tr>
<th>Results Window</th>
<th>Fig. 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td></td>
</tr>
<tr>
<td>Object</td>
<td>8.59</td>
</tr>
<tr>
<td>Area</td>
<td>2.15</td>
</tr>
<tr>
<td>Centroid</td>
<td>18.45</td>
</tr>
<tr>
<td>Centroid X</td>
<td>18.77</td>
</tr>
<tr>
<td>Centroid Y</td>
<td></td>
</tr>
<tr>
<td>Std. Dev.</td>
<td>4.41</td>
</tr>
<tr>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>10.52</td>
</tr>
<tr>
<td></td>
<td>10.14</td>
</tr>
<tr>
<td>1</td>
<td>#1</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>[6,1]</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>#2</td>
</tr>
<tr>
<td></td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>[20,2]</td>
</tr>
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<td></td>
<td>20</td>
</tr>
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<td></td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>#3</td>
</tr>
<tr>
<td></td>
<td>18.4</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>[26,3]</td>
</tr>
<tr>
<td></td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>#4</td>
</tr>
<tr>
<td></td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>[34,3]</td>
</tr>
<tr>
<td></td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>
Some measurement features are abbreviated in the column headings of the Results window. These include Max. Curv. (maximum curvature), ABR (ratio of the object's area to the area of the smallest bounding box), WLR (ratio of width to length), LWR (ratio of length to width), and FD0-FD7 (Fourier Descriptors 0 through 7).

### 4.2 Find Objects settings

ImageTool’s **Find Objects** tab (**Settings > Preferences > Find Objects**) contains settings that relate to the process of identifying objects in a thresholding procedure (Fig. 9). All features in the Find Objects preference page are controlled by code in Uthscsa ImageTool v. 1.27 (Wilcox et al. 1997), and not by CMEIAS. Consult the *It.doc* operator manual for pertinent additional information.

#### 4.2.1 AOI (Area of Interest) Options

**4.2.1.1 Search entire image**

When checked, ImageTool will look for candidate objects in the entire image. This option should **not** be checked if the image contains a magnification bar scale, otherwise this “invalid object” will be regarded as an object and be included in the object analysis.
4.2.1.2 Search in AOI

When checked, ImageTool will ask you to select an AOI. This is done before the thresholding stage by using the pencil cursor to draw a polygon enclosing the objects of interest (see section 5.7). When the "Include objects at edge of image" feature is deselected (described below), only those objects located completely within the select area of this polygon will be identified and analyzed. Also, Search in AOI must be checked when the image contains a bar scale so the polygon can be drawn to exclude it from object analysis.

4.2.2 Search options

4.2.2.1 Manually select objects

Check this feature to manually select the objects to be analyzed from those automatically identified by the Find Objects command. While ImageTool will still find all of the objects for you, it will only analyze the objects you choose. Use this feature to analyze only some segmented objects of interest in an image that also contains many invalid objects, or if the number of objects of interest in the thresholded image exceeds the 498 limit (see also sections 4.2.2.5 and 5.8).

4.2.2.2 Automatically select objects

If checked, ImageTool will analyze all objects found in the image that satisfy the constraints specified below. This feature is commonly selected when using CMEIAS.

4.2.2.3 Include objects at edge of image

If checked, then automatic object selection will retain and analyze objects that touch any edge of the image or AOI polygon. This option is off by default, since the border objects may be incomplete, so further size- or shape-analysis of them can be erroneous. Deselect this feature when using CMEIAS to analyze cell size and morphotype classification. Also, you must carefully consider whether partial objects located at the edge of the image should be counted. You can add a little extra, noise-free background margin to an image while editing it (image > [larger] canvas size in Photoshop) to facilitate drawing the AOI polygon and avoid excluding important objects during the Find Objects routine in image analysis. For object counting purposes, cells touching 2 edges of the image (e.g., bottom and right) should be counted, and those touching the other 2 edges should be excluded from the count.

4.2.2.4 Exclude background

If checked, ImageTool will attempt to identify the region of the image that is background, and will not consider it an object in automatic object selection. ImageTool considers an object to be background if its width and height are at least 90% of the image size. Use this feature setting when analyzing images using CMEIAS plugins.
4.2.2.5 Maximum # of objects

Unfortunately a coding error exists in the host program, ImageTool v. 1.27 that prevents the Results window from displaying more than 498 rows. So if 499 or more objects are found in an image, the Results window will only report on data extracted from the first 497 objects plus the last object found. Data on the latter object will be located in the row designated for object # 498. This display problem is illustrated in Fig. 10, where an image containing 1,148 bacteria was analyzed.

![Image of Table and Image](image)

**Fig. 10.** Display bug in the Results window of object analysis using ImageTool v. 1.27 when more than 498 objects are found in the image’s area of interest.

This problem is a bug in the Image Tool v. 1.27 code rather than by design, and developers of ImageTool have fixed it in release versions of Image Tool v. 2.X and higher, but CMEIAS v.1.27 plugins can only operate in ImageTool v. 1.27. Thus, CMEIAS v. 1.27 users should avoid this display problem by setting this field to 498 objects (Fig. 9) to prevent the loss of important image analysis data. Since individual images intended for CMEIAS morphotype analysis should ideally contain no more than 170 microbes anyway (to avoid excessive crowding, see section 3.1), it may be necessary to dilute the sample, acquire new images in microscope fields containing fewer microbial cells, redraw the AOI polygon to specify smaller portions of the images, and/or use the Manually Select Objects mode to avoid exceeding the 498 upper limit (see sections 3.1, 3.3, 4.2.1.2, 5.7 and 5.8 for more details). If the number of objects found in an image exceeds the limit specified in this edit box, an information box will display instructing the user to specify a larger number.

4.2.2.6 Minimum size of objects (pixels)

This field defines the minimum number of pixels of an object in order for it to be automatically found and included in an analysis. Objects containing fewer pixels than specified will be excluded by automatic object selection. **Note:** when optimally defined, this feature can efficiently eliminate small invalid objects and image background noise during the brightness threshold routine. This is done by first performing a manual area analysis (Analysis>Area) of the smallest object of interest in the image using the default units of pixels (see sections 5.6 and 5.8 on Calibrate Spatial Measurement and Manually Select Objects, respectively), and then by specifying a slightly lower value in this
**Minimum Size of Objects** field so all smaller objects of image noise are automatically excluded. No "maximum size of objects" is currently featured in IT.

When a CMEIAS morphotype classification is performed, this setting of minimum size (pixels) must be at least **30 pixels per object** (Fig. 9). This satisfies the requirement of sufficient magnification and pixel sampling density needed for CMEIAS to accurately define the object’s contour and shape (see section 3.1). Objects containing less than 30 pixels commonly have exaggerated, jagged edges (since the pixels are large relative to the object itself), often causing CMEIAS to misclassify certain morphotypes (e.g., regular rods will commonly misclassify as prosthectes). The setting of minimum size can be < 30 if objects are only counted and analyzed but not classified morphologically. If necessary, follow the procedure described in section 3.6 to increase the image’s pixel resolution before image analysis and morphotype classification.

### 4.2.3 Display options

#### 4.2.3.1 Show object count in a message box

When checked, ImageTool will display a message box containing the number of objects found by the threshold operation. Select this feature only when needed since it adds an interactive step (you must click the "OK" button) to complete each image analysis cycle. This feature does help to keep track of the cumulative number of objects analyzed and to avoid the 498-object-analysis-limit problem when concatenating object analysis data in the Results window (see Maximum Number of Objects in section 4.2.2.5).

#### 4.2.3.2 Place object count in Results window

Check this option if (and only if!) you want the Results window to indicate the number of objects found in the image after a threshold operation (Fig. 9). If the Results window already contains data from a previous image analysis, a dialog box will automatically prompt, asking if you want to save these previous data. A “yes” response will display a second dialog box to enter the name and location of the *.txt file to be saved. **Note:** regardless of the answer selected, the data in the Results window from the analysis of the previous image will be cleared automatically when this option is selected. Therefore, do **not** select this feature if you want to **concatenate** the object analysis results of multiple images comprising the same dataset (described next). Furthermore, object analysis data in the Results window always include a column of object numbers (Figs. 8, 10), so the object count in the image can always be obtained from that data list.

#### 4.2.3.3 Concatenate Object Analysis Results

Complex microbial communities cannot be fully represented by single microscopical images. Therefore, multiple images must be acquired and analyzed to build a representative image dataset of the community structure. When this is necessary, it is desirable to build a worksheet listing the object analysis data of each selected measurement feature for all valid objects in all images constituting the same dataset. CMEIAS/ImageTool v. 1.27 can directly perform this **concatenation** of data (up to the 498 object limit) in the Results window when the feature “Place object count in Results window” in the Find Objects page is **deselected** (Fig. 11). In this case, the rows of numbered objects analyzed for multiple images are concatenated, *i.e.*, the data extracted from each new consecutive image will be concatenated in the Results window.
Example of concatenated data in the ImageTool Results window: object analysis results of 2nd image listed just below 1st image containing 43 objects.

restart the numbering of objects in the Results window as “# 1”. The computed mean and standard deviation of all the measured values within each column of the Results window will automatically update as the new object analysis data of the most recently analyzed image are concatenated to the bottom of the listed data extracted from the previous image(s). Caution: when concatenating object analysis data, you must keep track of the cumulative number of objects from each of the consecutive images within the dataset. This is because, as indicated in section 4.2.2.5, the Results window of IT Ver. 1.27 only reports on the first 498 objects found. The cumulative number of objects analyzed in the data set is indicated in the cell of the leftmost gray column that also contains the “Mean / Standard Deviation” row labels and the last object row displaying its measured values (# 45 in Fig. 11). When the cumulative total approaches the 498 upper limit for reporting results, remember to cut (Edit > Cut Results) (not copy) these data from the ImageTool Results window and paste them into a compatible spreadsheet program so you don’t lose data by exceeding this upper limit of objects analyzed. Then continue performing object analysis on additional images in the same data set, and repeat this data transfer procedure as necessary [X ± std. dev. will not remain current].

4.2.3.4 Show object numbers on original image

If checked, ImageTool will place the ordinal number assigned consecutively to each object in the thresholded image as the scanning analysis proceeds from the bottom to the top of the image. Objects located along the same horizontal position in the image get numbered from left to right as illustrated in Fig. 12:

Fig. 12. ImageTool numbers each object found in the image from the bottom-up and from left-to-right along the same horizontal position.
This numbering of objects on the image matches the corresponding object number and associated object analysis data reported in each row of the Results window, and is also useful for interpreting the results of further analysis functions, allowing you to visually match object numbers with corresponding object analysis data. **Note:** the numbers are not actually part of the image itself, but are **annotations** that remain only while the image is opened. They will **not** appear on a direct printout of the image (File > Print Image), on an image saved directly in ImageTool (File > Save Image As), or copied (Edit > Copy Image) to another program that can accept it. To capture these colored annotations (plus the “object outlines” described in section 4.2.3.6) in the displayed image, click the “Print Screen” keyboard key to copy the entire monitor display to the clipboard, open an image editing program (e.g., Adobe Photoshop©), create a “New” blank image (File > New; default will be 72 dpi and RGB Color Mode), edit > paste the image, and then flatten and crop the area of interest from within the new image. Turn this "Show # s" feature **on** for CMEIAS image analysis.

### 4.2.3.5 Choose font

By double clicking this button, a dialog box appears to select the font type, style, color, and size to annotate object numbers. Try **14 pt Tahoma blue regular**.

### 4.2.3.6 Show object outlines on original image

If checked, ImageTool will place annotations onto the image showing the perimeter contour outlines of the objects it identified. As with the object numbers, these outlines are not made to the image itself, but they will be included in a screen capture image of the monitor display as described in section 4.2.3.4 above. This feature helps to determine if all the objects of interest that are to be analyzed have been found by the thresholding procedure, or if further segmentation of the image is required.

### 4.2.3.7 Choose Color

Double click this button to select the color used for the annotated object outlines, which can be the same or a different color than the object number. We suggest the bright **magenta** color illustrated in this example of a regular rod bacterium.

### 4.3 Image settings

ImageTool’s Image page (Settings > Preferences > Image) contains settings that control the manner in which images are displayed (Fig. 13).
4.3.1 Origin of coordinate system in image windows

The pixel locations in an image are defined by their unique Cartesian $x$, $y$ coordinates relative to a corner landmark origin. ImageTool’s default origin is the **Upper-left** corner (positive $y$ values go down). However, set the origin of coordinates to the **Lower-left** corner (positive $y$ values go up) when extracting georeferenced data. The coordinate of the origin point is controlled by the **Origin is (0,0)** and **Origin is (1,1)** buttons. These selections control how the $x$, $y$ coordinates of the mouse cursor on the image display in the status bar at the bottom of the ImageTool workspace. For CMEIAS analyses, set the landmark origin of coordinates to **Lower-left** and the origin point to **Origin is (0,0)**.

4.3.2 Prompt to save untitled images

If checked, ImageTool prompts you to save each new image created by the image processing tools before it is closed. Save color images as a bmp file.

4.3.3 Show full pathnames in image title bars

If checked, then the full filename for the image, beginning at the drive letter, will be displayed in the title bar of the image window. If not checked, then only the filename will be displayed. The latter selection is recommended when working with images that are too narrow to display the full filename.

4.3.4 Initial zoom/scale factor

These radio buttons are used to select the zoom factor when opening a new image. Set this at 1:1 unless you optimize otherwise. For example, open small (128x128) images at a zoom factor of 2:1, but set this to 1:2 or lower to scale down images that are wider or taller than your screen to make them fully visible when opened. This selection does not affect the zoom commands – you can still zoom in and out of the image as normal, it just affects the **initial** display of the images when opened.
CHAPTER 5

Image display adjustments, spatial calibration, and find objects thresholding in ImageTool

5.1 Load image

The image must be an 8-bit uncompressed grayscale for automatic object analysis. Load an image in ImageTool by one of 3 ways: click File > Open; click on the Open shortcut button (far left button in the toolbar); or click the F2 hotkey. This displays an Open window where you specify the appropriate drive, folder, and name of the image file to be analyzed, and click the Open button. The image window must be active (title bar colored) to perform subsequent steps. Consult the ImageTool Operator Manual It.doc file to work with stacked or tiled image displays.

5.2 Adjust image size

Use the Zoom In (+) [= F11 hotkey] or Zoom Out (-) [= Ctrl-F11 hotkey] buttons to display the entire image at the highest magnification that fits within the image window. The normal zoom range is 8:1 to 1:4. Useful Tip: To display very large images, use your mouse left button to apply multiple, rapid, repeated clicks of the Zoom out (-) button to bypass the 1:4 zoom out limit. For example, the very large image in Fig. 14 (a 416-inch wide montage image built from 26 geo-referenced scanning electron micrographs of bacteria colonized on a rice root) was zoomed out to 1:41 by this special procedure in order to show fully on-screen.

![Fig. 14](image-url)
5.3 Transformations

The image can be rotated to fit within the ImageTool workspace on screen by selecting Processing > Transformations > Rotate 90°, 180°, 270°, reverse, or flip.

5.4 Adjust image contrast / brightness

Use this ImageTool tool to interactively manipulate these features directly on the active image using slider bars before the image is thresholded. When needed, activate this Brightness / Contrast tool by selecting “Window > Show Contrast Window”, clicking the F7 hotkey, or clicking the Brightness / Contrast Tool shortcut icon (Fig. 15).

Fig. 15. ImageTool's feature to adjust image contrast and brightness, plus transform it to the negative image.

5.5 Negative image transformation

Selecting the “Negative Image” box in the Contrast/Brightness window (Fig. 15) inverts the grayscale brightness table, producing the corresponding negative image. Fig. 16 illustrates the use of this option to transform an original grayscale immunofluorescence micrograph that contain fluorescent bright bacteria against a dark background.
5.6 Calibrate spatial measurement

The default measurement unit for ImageTool is the pixel. Use the Settings > Preferences > Calibrate Spatial Measurements feature to spatially calibrate the image, i.e., automatically convert image pixel dimensions to the selected measurement unit for all dimensional analyses. It is valid only for the image in which the calibration is performed. First, open the image and zoom in on an object of known length to the highest magnification that can be fully displayed on-screen, e.g., a 10 µm magnification bar scale embedded in a remote corner of the image (Figs. 5 and 17). Select the Calibrate Spatial Measurement feature. A dialog box (Fig. 17) will display in ImageTool instructing you to draw a line of known length. When placed on the active image, the cursor will change to a pencil. Define the line by carefully positioning the pencil point at one end of the bar, then
click-and-drag the mouse cursor across the length of the bar, and release the cursor precisely at its opposite end. Alternatively, click with the pencil point at one end of the bar scale and double click with the pencil point at the other end. First-time users should practice doing this a few times to verify your accuracy. You cannot calibrate with a multi-segment line. The Calibrate dialog box will automatically display the number of pixels for the line drawn. Select the units of measurement, e.g., microns = µm or 10^-6 meter [renamed as “micrometer” in ImageTool v. 3.0], and then enter the bar scale length (in this example, “10.0”) in the white box (Fig. 17). The units must be chosen before a length value can be entered. Finally, click the OK button after you have confirmed the input. Cancel and repeat if necessary. All dimensional analyses performed on this image will be based on this spatial calibration. Useful tips: You can remove calibration from an image by selecting the Calibrate Spatial Measurement command, drawing a line of any length, and accepting the initial value in pixels. Since shape is a dimensionless characteristic independent of size, all shape measurement features required to perform the CMEIAS-2 morphotype classifier are unitless and therefore they can be computed in pixel units extracted from objects without additional spatial calibration. However, all dimensional image analyses (e.g., area) require spatial calibration.

The Settings > Load Spatial Calibration command loads previously saved spatial calibrations to be used in dimensional analysis. To create this *.itc spatial calibration file, click “yes” when the dialog box offers to save the calibration of an image that serves as the default magnification. Once loaded, this spatial calibration becomes the default for all measurement on all images, both those currently open and those opened later in the same image analysis session. The loaded spatial calibration remains in effect only until the end of the current image analysis session, or until another spatial calibration is loaded. To revert to the uncalibrated behavior, you must load the uncalibrated No Calibration.itc file located in the ImageTool \ Calibration folder.

5.7 Find objects by brightness threshold segmentation

The foreground objects of interest (e.g., bacteria) in the image must be found before they can be analyzed. In ImageTool, this is accomplished by threshold segmentation based on differences in grayscale brightness between the pixels of the foreground objects and the background. ImageTool considers an object to be background if its width and height are at least 90% of the image size. Using this threshold procedure, one can select the objects automatically (all of section 5.7) or manually (section 5.8). Successful thresholding of grayscale images to automatically select objects (Fig. 9) requires that all the foreground object pixels have brightness values outside the range of those that are background. Commonly, images require editing to fulfill this fundamental criterion for digital image analysis.

5.7.1 Activating threshold selections
To automatically find the objects in a user-defined Area Of Interest in an image, select Settings > Preferences > Find Objects > Search in AOI >Apply (Fig. 9). Then activate the threshold routine by selecting Analysis > Object Analysis > Find Objects or click the Find Objects shortcut icon to display its box (Fig. 18).
5.7.2 Find objects in a binary image

If the image is binary (has only black and white pixels with grayscale brightness of 0 and 255, respectively), select None [image is already thresholded] in the Find Objects dialog box (Fig. 18). If Find Objects > Search in AOI is selected, you will be instructed to draw a polygon on the image. Use the pencil cursor to draw a thin line polygon (left click at every corner) that includes all of the foreground objects to be analyzed but excludes the bar scale and associated text, (optionally) plus any other “invalid objects” present within the image (Fig. 18). Close the polygon automatically by double clicking to connect a final line from the current position of the cursor to the start position. The polygon line will be blue when located in open background areas of the image (Fig. 18) and a contrasting yellow color when it covers an object. If some objects of interest are located at the edge of the image, add a little extra white margin to the image canvas beforehand (e.g., in Adobe Photoshop: select white background, then Image > Canvas Size) so sufficient space is available to draw the polygon to enclose all of these foreground objects of interest. The image will then display with the annotated objects and a separate information box indicating the total number of objects found will also display if this feature is selected in the Find Objects page (section 4.2.3.1), see Fig. 19. If Find Objects > Search Entire Image is selected (section 4.2.1.1) instead of Search in AOI (e.g., when no bar scale is present), the thresholding procedure will find and annotate the objects in the binary image without using the polygon.

Fig. 18. ImageTool's threshold routine to find the image's foreground objects of interest.
5.7.3. Find objects in a non-binary image

If the grayscale image contains pixels of varying brightness (Fig. 20A), select the **Manual** thresholding method in the Find Objects box (Fig. 18) and click **OK**. Draw the polygon enclosing all foreground objects of interest as described above while excluding invalid objects and the bar scale if present. Closure of the AOI polygon will automatically fill it in red and open an ImageTool window displaying a histogram of the gray levels within the image (Fig. 20B). When positioned over the slider bar, the cursor changes from a white arrow to a black plunger. By slowly sliding the plunger to the left, pixels with gray levels whose brightness values fall between the two endpoints will become red while pixels outside the range (background) will revert to their original gray levels. Continue sliding the plunger position further to the left until all foreground objects are separated from background, and simultaneously, their red-filled contour accurately represents their size, shape, and position as in the original grayscale image (Fig. 20C). After thresholding the image, release the mouse button and click **OK**. The foreground objects will be numbered and their contours annotated in front of the original grayscale image background as illustrated Fig. 20D. Nice image!

The ImageTool download includes the image “blobs.tif” that works well to practice learning how to threshold non-binary grayscale images accurately.

*(space intentionally left blank)*
Fig. 20 A-D. The various steps to find the foreground objects of interest in a grayscale image using the ImageTool brightness threshold segmentation procedure.

Figs. 20A-D illustrate these threshold segmentation steps for a non-binary grayscale image of bacteria requiring no editing of background to fulfill this criterion. Fig. 20A shows the grayscale image that has been previously edited to split a few touching cells by drawing a white-colored line of single pixel width between them (arrows) using the pencil tool in Adobe Photoshop (see section 3.7). Fig. 20B displays the threshold window with the complex histogram of pixel brightness levels in this grayscale image. Fig. 20C shows the segmented objects (colored in red) found in this image by brightness thresholding to eliminate the background pixels. Fig. 20D shows the resultant thresholded image with each bacterial cell found displayed with an annotated contour and assigned number ordered from the bottom up. The interactive "slider" action for this thresholding procedure will be gradual for non-binary grayscale images, but will be quick and abrupt for binary images.
5.8 Manually select objects
In most cases, the image will be edited sufficiently so that all of the foreground objects of interest can be segmented from background by the threshold procedure using the Automatically select objects feature selected in the Settings > Preferences > Find objects page. This is the commonly used method. However, manual selection is preferred for some cases. ImageTool’s Manually select objects feature is a useful alternative to the automatic mode (see Fig. 9) when the image(s) contain invalid objects whose pixel brightness lies in the range that defines the foreground objects and/or only some of the foreground objects need to be analyzed, neither of which can be accommodated by the automatic thresholding routine. When this option is active, Image Tool will instruct you to select the objects to be analyzed from those automatically identified by the Find Objects command. Although ImageTool will still find all of the objects for you, it will only report image analysis data on those foreground objects of interest that you select and will exclude the rest, as illustrated in Fig. 21.

![Fig. 21. ImageTool's Manually select objects feature. In A, only 4 of the objects found (# 42, 51, 52, and 55) are manually selected as indicated by being temporarily filled with the magenta color selected for the contour annotation. After clicking the “Done” button (B), these manually selected objects are reassigned object numbers (C) and are registered as such in the Results window (D) after an object analysis.](image-url)
Performing Object Analysis

6.1 Overview of settings, preferences, and segmentation of objects

Before performing object analysis, you must specify the various Find Objects and Image preference settings (Settings > Preferences > Find Objects: area of interest, search, display, minimum pixel size; > Image: Origin of Coordinates, Initial Zoom setting), select the appropriate measurement feature(s), specify the decimal precision to report data, load and spatially calibrate the image, and segment the image using the interactive brightness threshold procedure to find the objects of interest. See sections 4.1 – 4.3 of this operator manual for details on these routines.

6.2 Object Counting

ImageTool provides 6 different ways to obtain the number of foreground objects in the thresholded image; 5 of these are counted automatically and 1 is counted manually.

6.3 Automatic object counting

Fig. 22 illustrates 3 ways ImageTool displays the object count automatically. First, if Settings > Preferences > Find Objects > “Show object numbers on original image” is selected (section 4.2.3.4), the object count will equal the largest annotated number assigned to the highest positioned object found in the image (69 in this example, see arrow). This method is useful if neighboring objects do not obscure the largest numbered annotation. Second, if Settings > Preferences > Find Objects > “Show object count in a message box” is selected (section 4.2.3.1), then immediately after thresholding, ImageTool will display a dialog information box indicating the number of objects found in the thresholded image’s AOI (Fig. 19 and 22). Third, if Settings > Preferences > Find Objects > “Place object count in Results window” is also selected (section 4.2.3.2), the
mean and standard deviation of object counts for all images in a counting session including the object count of the current image will be displayed in the Results window (Fig. 22). Since in this example only one image was analyzed, the Std. Dev. is 0.00. If the dataset for counting objects consists of multiple images, you can concatenate the object count data for each image by deselecting the “Show object count in a message box” option in the Find Objects tab (Fig. 9, section 4.2.3.3). The mean and standard deviation for the dataset will update in the Results window with each new image analyzed (see section 4.1.4). The 4th and 5th ways to obtain the object count automatically are featured in the object analysis and object classification routines described later in sections 6 and 7.

Fig. 22. Automatic object counting in ImageTool. The total number of foreground objects in the image is indicated by: (1) the top annotated object (arrow), (2) the information box, and (3) the Results Window.

6.4 Manual object counting

ImageTool provides a manual object counting feature (select Analysis > Count and Tag or the corresponding Toolbar shortcut) for both grayscale and colored images (Fig. 23). This feature is useful when all of the objects of interest in a grayscale image cannot be segmented automatically, or if a color image is being analyzed. When this feature is selected, the mouse cursor becomes a pencil when placed over the active image. Point to each object of interest and count it by a single click of the left mouse button. This procedure registers a colored dot on the object counted in the image (to indicate it has already been counted) and the current incremental count in the Tag & Count dialog box. Conclude the counting process by double clicking the final object to be counted (this clarifies a bit of confusion in the ImageTool Tag & Count box instructions). The radius size (in pixels) and color of the dot register can be specified in the Settings > Preferences > Object count tab page. The object count will display in the dialog box and be sent to the Results window as illustrated in Fig. 23.
Now that you have found the foreground objects of interest, follow these steps to perform an object analysis on them.

1. Select any combination of measurement features (Fig. 6; Settings > Preferences > Measurement Features selection page) that you want CMEIAS/ImageTool to extract from the foreground objects found. The 7 shape measurement features that must be selected in the object analysis routine used by the CMEIAS morphotype classifier are conveniently enclosed within a framed box in the measurement feature selection page (see section 4.1.1, Fig. 6).
2. Activate the most recent thresholded image. Spatially calibrate the image only if measurement attributes must be reported in user-defined units (unnecessary for shape measurements since they are dimensionless).
3. Click on Analysis > Object Analysis > Object Analysis to extract the selected measurement features from each object in the image. The computing time required for this step will vary.

Fig. 23. Manual Count and Tag feature in ImageTool Ver. 1.27.
depending upon the speed of the computer, the number of thresholded objects in the image, and the combination of measurement features selected. The computing time required to perform object analysis on the binary image used to make Fig. 24 took between 1-2 seconds on a Pentium III PC running at 700 MHz.

![Image](image.png)

**Fig. 24.** Object analysis and display of data in the ImageTool Result window. Note that the object count in the image is indicated by the highest numbered row of object analysis data (arrow) in the Results window corresponding to the highest object found in the image (arrow).

4. Following object analysis, each selected measurement feature appears as a **column heading** in the **Results window** spreadsheet (Fig. 24, also see section 4.1.4 and Fig. 8), and the corresponding values extracted from each foreground object found in the image are reported individually in rows in units that are designated during the “calibrate spatial measurement” step (see section 5.6). The mean and standard deviation for all measured values in each column array are automatically computed and displayed in gray filled cells of the first 2 rows above the first object row of data (Figs. 8 and 24). This object analysis routine is the 5\textsuperscript{th} way to determine the number of objects in the image [4\textsuperscript{th} automatically], since that value equals the highest numbered row of data in the object analysis Results window (see black arrow in Fig. 24).

The only exception to this standard display design of the Results window is in the reporting of **Centroid X|Y** and **Gray Centroid X|Y** data. When selected, these attributes display 3 columns of image analysis data for each object found: the ImageTool “Centroid” contains both the \(x\) and \(y\) coordinates delimited by a comma in the same worksheet cell, and CMEIAS lists these “Centroid X” and “Centroid Y” coordinates in separate cells (Fig. 8), in pixel units. This alternate display
facilitates the export and use of these spatial coordinates in other software applications for object spatial distribution analysis.

6.6 Working with Object Analysis Data in the Results Window

I. Five options are available for the object analysis data in the Results window:
   
a. Manually edit individual selected cells using the numerical keyboard.
   b. Edit >Copy Results to the clipboard and paste into a Windows-compatible worksheet.
   c. Edit >Cut Results to the clipboard and paste into a Windows-compatible worksheet.
   d. Edit >Clear Results: clears the data from the Results window. When selected, you are first prompted whether or not you wish to save the current results.
   e. Minimize Results window and proceed to perform an Object Classification of the same image or an object analysis of a new image. In the latter case, decide beforehand whether or not to concatenate the object analysis data [see section 4.2.3.3].

Note: ImageTool minimizes (rather than closes) the Results window when either the “—” or the “X” delete button in its upper right corner is clicked. This wise design prevents unintentional loss of data.
Performing Object Classification

Microscopy commonly reveals various characteristics of bacteria relevant to classification of their morphological diversity in *actively growing* microbial communities. Such diversity can be enormous as illustrated in the bovine rumen microflora (Fig. 1, the image that created the spark of inspiration for CMEIAS), and CMEIAS is designed to extract the information in such community images so that morphological diversity can be quantified. In contrast, morphological analysis of *non-growing* microbial communities is much less informative since most of the microbes have differentiated into dwarf, nearly spherical, quiescent *ultramicrocells* for starvation survival, an ecologically important physiological adaptation whereby the cells shut down their cell wall-building machinery for their cell division cycle and their particular morphotype while actively growing.

CMEIAS® uses various measurement features and two object classifiers to extract size and/or shape attributes of microorganisms in segmented, digital images of microbial communities and classifies them into their appropriate morphotype. These supervised object classifiers report on the richness of different morphotypes found within the images and the distribution of abundance among each of them, thus providing the ecological data needed to compute various morphological diversity indices of microbial communities.

The preference page (Settings > Preferences > Object Classification) to select the two CMEIAS / ImageTool v1.27 object classifiers and associated features is illustrated in Fig. 25. Options include selection for the type of Object Classifier, report on objects individually (see details on this feature in the ImageTool 1.27 *It.doc* operator manual, not very useful here for CMEIAS v.1.27), and display a new image with objects pseudocolored according to their assigned classification.
7.1 ImageTool / CMEIAS-1 Object Classifier

The first object classifier sorts microbes in up to 16 bin classes based on a single, user-selected attribute of size or shape and user-defined upper class limits. Since only a single measurement feature is used, one can perform this object classification without prior object analysis of the image. This object classifier can classify cell shapes in relatively simple communities of bacteria containing only a few morphotypes (e.g., regular rods, cocci, and filaments), and is adequate for size classifications of all bacteria in the image, regardless of shape.

ImageTool Ver. 1.27 designed the first object classifier to sort objects based on similar measurement characteristics. The user selects the single measurement feature and number of groups (called bin classes, between 2 to 16), then enters the upper class limit values for each bin except the last one, which will group objects whose attribute value is greater than the largest upper class limit entered. The bin widths do not have to be constant. The output from this object classifier is a listing of each bin range, corresponding object counts for each bin, and the mean and std. dev. of measurement values in each bin. The measurement features added in the CMEIAS code makes this first object classifier adequate for relative size classifications of all bacteria in the image regardless of shape, and for analysis of the morphological diversity of certain relatively simple communities containing only a few morphotypes (e.g., regular rods, cocci, filaments). Since the object classification is performed directly, a separate prior object analysis is unnecessary. The usefulness of this simple object classifier is illustrated in Figs. 26 and 27, where cells in the image are classified into bins according to their optimized size (area) and shape (width : length ratio) attributes, respectively.
Fig. 27. Morphotype classification of cocci, regular rods, and unbranched filaments using the ImageTool/CMEIAS-1 Object Classifier with the Width : Length Ratio measurement feature and optimized upper class limits of 0.0625 and 0.5. Classification data are reported for each bin class.
7.1.1 Using the IT/CMEIAS-1 Object Classifier for cell size analysis

1. (Fig. 26) Set Settings > Preferences as follows: Find Objects page (Fig. 9): Search in AOI, Automatically select objects, Exclude background, Show object numbers on original image, Show object outlines on original image. Object Classification page (Fig. 25): Report on classification using a single measurement feature, Display new image showing objects colored by classification.
2. Open and spatially calibrate the image to be analyzed (see Fig. 17, section 5.6).
3. Select Analysis > Object Analysis > Find Objects, and perform the brightness threshold routine (see Figs. 18-21 and section 5.7) to find all the foreground microbes of interest in the image. There are 170 microbes in the CocciRegularRodFilament.tif image used to make Figs.26-27.
4. Select Analysis > Object Analysis > Object Classification to open the CMEIAS / ImageTool-1 Define Objects Classifications window (Fig. 26).
5. Click the Attribute pulldown box and select the measurement feature desired (Area in Fig. 26).
6. Enter the upper class limit for each bin in the Maximum Value in Class fields (Fig. 26). Alternatively, click the Load button, select an appropriate *.ocd classification file previously recorded and saved in the Uthscsa\ImageTool\Calibration folder, and click Open. (ImageTool prompts you to save any new set of upper class limit values as an option). All appropriate fields of the Maximum Value in Class will be filled automatically with the upper class limits values.
7. Verify that the Attribute selection hasn't been changed (it does this sometimes when loading a classification file), and then click OK to perform the object classification.
8. The pseudocolor classification image and classification data will display onscreen (Fig. 26).

7.1.2. Using the IT / CMEIAS-1 Object Classifier for cell shape analysis (Fig. 27)

1. Perform above steps 1, 3-8 (for #5, select shape attribute, e.g., width/length). Since this shape classification only recognizes 3 morphotypes (coci, regular rods, and unbranched filaments), just 2 fields of the Maximum Value in Class are entered. The 3rd bin will include all objects whose attribute is higher than the 2nd upper class limit value entered. Spatial calibration of the image is not required for dimensionless shape measurement attributes.
2. The newly created classification image and the classification data will display onscreen.

7.2 CMEIAS-2 Morphotype Classifier

A second CMEIAS hierarchical tree classifier uses an optimized subset of multiple shape measurement features to analyze significantly more complex communities containing greater morphological diversity than ever before possible. This comprehensive CMEIAS® shape classifier uses a series of pattern recognition algorithms optimized by us to automatically classify each bacterium into one of 11 predominant microbial morphotypes distinguished by a hierarchy of shape characteristics outlined in Fig. 28. This set of 11 morphotypes equals the morphological diversity represented by 98% of the genera in the 9th Edition of Bergey’s Manual of Determinative Bacteriology. Optimization of the pattern recognition algorithms used by CMEIAS are described in Liu et al. 2001 Microbial Ecology 41: 173-194 and 2001 Microbial Ecology 42: 215.

To produce a CMEIAS-2 Morphotype Classification, CMEIAS first extracts the required shape attributes (7 selections enclosed within a framed area of the Measurement Feature page) from each object in an object analysis of the image, and then applies these shape analysis data to the pattern recognition algorithms to perform the supervised classification of each object's morphotype. ☺
7.2.1 & Fig. 28. Hierarchy outline of characteristics for microbial morphotypes classified by CMEIAS v 1.27. The class labels and pseudocolor assignments (against a black background) for each morphotype are also indicated. (The pseudocolored objects are brighter on screen than in print).

<table>
<thead>
<tr>
<th>Morphotype Classification Criteria</th>
<th>Label</th>
<th>Morphotype</th>
<th>Pseudocolor Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.  Rounded cells (spheroid, ovoid, coccobacilli)</td>
<td>A</td>
<td>COCCI</td>
<td><img src="image1" alt="Image" /></td>
</tr>
<tr>
<td>II.  Elongated cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.  Unbranched (2 cell poles)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Repeated waveform</td>
<td>B</td>
<td>SPIRAL</td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>2. Single regular curvature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Curvature is vibroid or crescent</td>
<td>C</td>
<td>CURVED ROD</td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>b. Curvature is U-shaped</td>
<td>D</td>
<td>U-SHAPED ROD</td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>3. Linear (no repeated curvature of the medial axis)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Constant cell width</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Length / Width ratio is &lt; 16:1</td>
<td>E</td>
<td>REGULAR ROD</td>
<td><img src="image5" alt="Image" /></td>
</tr>
<tr>
<td>2. Length / Width ratio is &gt; 16:1</td>
<td>F</td>
<td>UNBRANCHED FILAMENT</td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>b. Widest at center, tapered at both poles</td>
<td>G</td>
<td>ELLIPSOID</td>
<td><img src="image7" alt="Image" /></td>
</tr>
<tr>
<td>c. Wider at one pole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Gradually tapered at opposite pole</td>
<td>H</td>
<td>CLUB</td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>2. Thin stalk appendage</td>
<td>I</td>
<td>PROSTHECATE</td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>B.  Branched (&gt; 2 cell poles)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Length / Width ratio &lt; 16:1</td>
<td>J</td>
<td>RUDIMENTARY BRANCHED ROD</td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td>2. Length / Width ratio &gt; 16:1</td>
<td>K</td>
<td>BRANCHED FILAMENT</td>
<td><img src="image11" alt="Image" /></td>
</tr>
</tbody>
</table>
7.2.2 Steps to perform a CMEIAS-2 Morphotype Classification

1. Set Settings > Preference pages as follows: Measurement Features (Fig. 6): check all 7 attributes within the Morphotype Classifier frame (Roundness, Elongation, Compactness, Max. Curvature, Width/Length, Area/BB Area, Fourier Descriptors). Find Objects (Fig. 9): Search in AOI, Automatically select objects, Exclude background, Show object numbers on original image, show object outlines on original image. Deselect Include objects at edge of image. Object Classification (Fig. 25): Report on CMEIAS morphotype classifier using multiple measurement features, Display new image showing objects colored by classification.

2. Open the image, select Analysis > Object Analysis > Find Objects, and perform the brightness threshold routine (section 5.7 inclusive) to find all the foreground microbes of interest in the image. There are 170 microbes in the example image, Community-A in Fig. 29.

3. Select Analysis > Object Analysis > Object Analysis to perform the object analysis on the image. Within a few seconds, the values of the various shape attributes will be extracted from each numbered object found and displayed in the Results window.

4. Make the same image active and then perform the object classification (Analysis > Object Analysis > Object Classification). This should take only a second or less of computing time.

![Figure 29: A) thresholded, binary, annotated composite image; B) reconstructed “classification result image” with each foreground object pseudocolored according to its morphotype class assignment against a black background; C) classification Results window displaying each class label, corresponding morphotype name, and object count for each class.](image-url)
7.2.3 Important points regarding the CMEIAS-2 Morphotype Classifier:

1. **Speed of operation**: Using a PC with Pentium II operating at 300 MHz with 384 MB RAM and 32 MB video card, the algorithms for object analysis and object classification presented their results for most of the images used in CMEIAS® development in an average of 2.0 sec and 0.2 sec per image, respectively. These durations are well below the approximate 10 sec “limit of user irritation”. Most of this time is actually used to produce the graphical display rather than computation *per se*, and of course, will be even shorter duration for faster computers. Awesome.

2. **Use of classification pseudocolors**: Rather than just being "cosmetic", each pseudocolor was selected with great care so it can easily be associated with the specific morphotype classification for each object (Fig. 29D + E). Once learned, this pseudocolor recognition scheme becomes an efficient tool to inspect the accuracy of the automatic morphotype classification and edit the results if necessary.

3. **Generate Classification "Out" file**: CMEIAS also automatically generates and saves a small text file of morphotype classification results in the same directory where the image file is located. This text file contains a numbered list of morphotype label classifications for each individual object (corresponding to its annotation number in the thresholded image), and is named the same as the corresponding image file followed by an “out” file extension. This *.out file is an automatic backup of the classification result but it can be deleted if so desired.
4. **Classification accuracy:** Extensive testing (Liu *et al.* 2001 Microbial Ecol 41:173-194) indicates that the CMEIAS-2 morphotype classifier performs with an overall accuracy of 97.0% on properly edited images, indicating that accurate classification of the rich morphological diversity in complex microbial communities is now possible (Table 1).

<table>
<thead>
<tr>
<th>Morphotype</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>Total</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Coccus</td>
<td>1212</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1217</td>
<td>99.6</td>
</tr>
<tr>
<td>B Spiral</td>
<td>0</td>
<td>399</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>408</td>
<td>97.8</td>
</tr>
<tr>
<td>C Curved Rod</td>
<td>0</td>
<td>0</td>
<td>156</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>164</td>
<td>95.1</td>
</tr>
<tr>
<td>D U-Shaped</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>132</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>148</td>
<td>89.2</td>
</tr>
<tr>
<td>E Regular Rod</td>
<td>24</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>110</td>
<td>4</td>
<td>1</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1158</td>
<td>95.0</td>
</tr>
<tr>
<td>F Unbranched Filament</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>240</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>243</td>
<td>98.8</td>
</tr>
<tr>
<td>G Ellipsoid</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>130</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>137</td>
<td>94.9</td>
</tr>
<tr>
<td>H Club</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>298</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>309</td>
<td>96.4</td>
</tr>
<tr>
<td>I Prostecate</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>206</td>
<td>100</td>
</tr>
<tr>
<td>J Rudimentary Branched</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>138</td>
<td>0</td>
<td>150</td>
<td>92.0</td>
</tr>
<tr>
<td>K Branched Filament</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>130</td>
<td>100</td>
</tr>
</tbody>
</table>

**7.2.4 Sources of Error & Edit Feature for CMEIAS-2 Morphotype Classification Results**

Extensive testing of microbial communities from the bovine rumen, glucose-fed anaerobic bioreactors derived from domestic sewage sludge, gut luminal fluid of *Reticulitermes flavipes* termites, biofilms from human dental and tongue surfaces, legume root nodules, and soil indicates that CMEIAS classifies microbial morphotypes with a cumulative error rate of \( \leq 3\% \) (Liu *et al.* 2001). CMEIAS makes three main types of errors when classifying microbial morphotypes in images that have adequate pixel sampling density and are properly segmented. The most common, type-1 error occurs when the cell’s shape lies in the real-world continuum at the border between the 14-dimensional space that defines each morphotype, and it visually fits better into a morphotype class other than the one assigned by CMEIAS. For example, although the CMEIAS classification of coccobacilli as cocci is well justified microbiologically and geometrically (Liu *et al.* 2001), the user may prefer that they be classified as (short) regular rods. A less frequent type-2 error results when the microbe has a very rare shape that does not match any of the 11 predefined morphotype classes in...
the CMEIAS-2 Morphotype classifier. The **type-3** error (made infrequent by careful image acquisition, editing and thresholding of the image prior to analysis) occurs when an interactive brightness threshold routine includes an object as foreground even though it actually is inanimate debris, a lysed cell fragment (e.g., ghost cell), or an invalid object of background noise that should not be included in the analysis.

To make the CMEIAS-2 Morphotype Classifier more flexible and reduce unwanted noise in the final data output, a plug-in module was implemented in CMEIAS v. 1.27 to permit interactive editing of the classifier results. This CMEIAS® edit module addresses each of these three major types of classification errors that occur during the automated morphological analysis of microbial communities. In this routine, the user inspects the assigned morphotype of each microbe based on visual recognition of its distinctive, classification-based assigned pseudocolor in the result image, then activates the edit feature, manually selects the specific object of interest, reassigns its appropriate morphotype class, and/or adds up to five user-defined “other” morphotypes to the classification scheme (details in sections 7.2.5 and 7.2.6). Once done, this interactive editing routine produces an automatic update of the corresponding morphotype frequencies in the classification Results window. A significant effort was made in development of this CMEIAS® edit plug-in module to select the pseudocolors that are rapidly distinguished from one another in the same image and are easily associated with the corresponding morphotype, so that interactive recognition of the morphotype classification results becomes almost instantaneous with user experience (Fig. 29D + E).

The steps to use this CMEIAS Morphotype Classification Edit module to address the first, main type of classification error are illustrated in Fig. 30. The CMEIAS-2 classifier automatically assigned a coccus morphotype (red pseudocolor) to the microbe found in the center of the image (white arrow) because of its high values of roundness and circularity plus length to width ratio of less than 2:1 (Fig. 30A). Such microbial morphotypes are common in many unamended soil and oligotrophic aquatic habitats. The classification edit feature was then used to reassign it's morphotype class to a regular rod (blue pseudocolor) (Fig. 30B + 30C), and when done, the morphotype classification frequency count data updated with the corresponding changes incorporated (Fig. 30D; cocci 62 → 61; regular rod 51 → 52). It’s handy for beginners to have a color copy of the Fig. 28 outline near your computer monitor while learning this CMEIAS Edit Classification routine. Once you’ve mastered the color ↔ morphotype recognition linkage, this interactive editing routine becomes efficient and as easy as taking candy from a baby. ☺
7.2.5. Steps to edit the type-1 misclassification error in a CMEIAS-2 Morphotype Classification

1. Carefully inspect the morphotype assignments of each object in the pseudocolored Classification result image (consult Fig. 28 until this inspection becomes instantaneous recognition with usage). Decide which object(s) need editing and the morphotype to which it should be reassigned.

2. Select Analysis (Main Menu) > Object Analysis > Edit Classification Results to activate the edit module. This will evoke a Reassign Category Label window with features grayed out.

3. Activate the pseudocolored Classification result image. Left-click the target object with the tip of the magic wand cursor (shown next to “Other-2” class and below label “B” in Fig. 30B). The object will blink when selected (a useful feature especially when objects have crowded
neighbors), and its pseudocolor will indicate its morphotype assigned by the CMEIAS classification.

4. The **Reassign Category Label** window will activate and display the radio button checked next to the selected object's currently assigned CMEIAS morphotype. In Fig. 30B, this is a coccus.

5. Reassign the object's morphotype by clicking its corresponding alternate radio button. When registered, the pseudocolor of the selected, blinking object in the Classification result image will automatically change to reflect the morphotype classification reassignment. In Fig. 30A + 30C, the cell has changed from red to blue, indicating it has been reassigned as a regular rod (Fig. 30B).

6. Repeat steps 1-5 for any other objects whose morphotype classification needs reassignment.

7. After all classification edits are made, click the "**Done**" button in the dialog box to accept the editing results. Before these are displayed, however, another window will pop-up, asking if you wish to save the original (unedited) data currently in the Results window. Most often, the appropriate response is "**No**" when they contain classification errors and you plan to copy the edited classification data to a spreadsheet program anyway. A "**Yes**" response will display another window instructing you to provide the name and location of the file of original data to be saved.

8. After entering your preferred response, the classification data in the Results window will update, reflecting the change(s) you made in the edit routine (in Fig. 30D, the frequency counts for cocci and regular rods are adjusted by one unit).

9. Copy the morphotype classification data in the Results window to a spreadsheet program for storage and analysis.

10. (Optional) You can copy the classification result image to the clipboard (Edit > Copy Image or printscreen) and paste it into an application that can accept it (e.g., Adobe Photoshop).

### 7.2.6 The type-2 (unrecognized class) and type-3 (invalid object) errors in a CMEIAS-2 Morphotype Classification, and steps to edit them

Accommodation of the type-2 error has the greatest impact in expanding the range of microbial communities that can be analyzed by CMEIAS®. This was accomplished by adding five other user-defined categories of bacterial shape (labeled as L through P corresponding to “Other-1” through “Other-5”) in the CMEIAS editing module interface, and assigning a unique pseudocolor to each of them (Fig. 29C). We considered this level of flexibility to be sufficient to handle most all communities, since only rarely would a community have a morphological diversity represented by more than 16 bacterial morphotypes at any one time. Also, since the morphotypes included in the CMEIAS classification scheme include all those that are common plus most that are uncommon (equal to the morphotypes represented by 97% of all prokaryotic genera in Bergey's 9th Ed. Manual of Determinative Bacteriology), only on rare occasions will the type-2 error occur in microbial community analysis. It has only occurred once among several years of community analysis by developers of CMEIAS. Furthermore, the rarity and unique character of other microbial morphotypes make them easy to find. These characteristics justify the interactive/manual design of this CMEIAS classification edit feature. Eliminate the type-2 error in morphotype classification results as follows:

1. Follow steps 1-4 in section 7.2.5 above to activate the edit feature module and select the object of interest whose shape matches none of the morphotypes classified automatically by CMEIAS.
2. Reassign the object (and all others of the same unique shape) to class L "Other-1".
3. Repeat steps 1-2 for any other object(s) of other unique shape(s) to class M "Other-2", then class N "Other-3", etc. Up to 5 "other" unique shapes can be added to the classification result.
4. Take note of a descriptor for each Other-X morphotype reassignment.
5. When all morphotype classification editing is complete on the image, click "Done" and copy the updated data in the Results window to a spreadsheet application.

6. In the pasted worksheet, click the column heading labeled "Other-1", etc., and rename the morphotype appropriately.

To illustrate the type-2 error, we evaluated 17 valid-but-rare bacterial shapes that are not included in the CMEIAS® Morphotype Classifier (Fig. 31; Liu et al. 2001). Accommodation of the first 16 bacterial shapes (labeled A-P) in community images only required the second editing scenario described above. Recognition of the pseudocolor assignments directly indicates that CMEIAS® misclassified them as follows: A as an ellipsoid; B and P as unbranched filaments; C as a prosthecate; D as a spiral; E and F as cocci; G as a curved rod; H as a regular rod; I-L as clubs; and M-O as branched filaments. The reasons for most of these classifications are fairly logical in the hierarchy classification scheme (see Fig. 28).


The last valid bacterial shape is a special type of unbranched filament that tends to coil, forming enclosed loops on the 2-dimensional projected image (objects Q and R of Fig. 31; resembles a cooked string of spaghetti). This coiled unbranched filament is the only morphotype in Fig. 31 that has both type-2 and type-3 errors requiring the second and third editing tasks to classify correctly. CMEIAS regards object Q as two objects, one (a spiral) represented by the outer contour of the cell and the other (a coccus) represented by its inner contour (= outer contour of the closed loop). These classification errors are corrected by first reassigning the internal loop as an invalid object (eliminating the type-3 error, assigned a very dark blue pseudocolor that is barely discernible from the black background), next reassigning the cell object as an “Other-1” morphotype, and finally renaming it as a Coiled Unbranched Filament after exporting the classification data to a compatible spreadsheet program.
Graphics and Ecological Statistics of CMEIAS object classification data

Here are examples of various graphics and ecological statistics performed on IT / CMEIAS-1 cell size and CMEIAS-2 morphotype classification data (Fig. 32A-32B, Tables 2-3) that are appropriate to include in microbial community analysis, using the Community A and Community B examples from Figures 29 and 30, respectively. The CMEIAS data were analyzed by ecological statistics using Howard Towner's EcoStat Trinity Software http://www.trinitysoftware.com/lifesci/index.html. For other examples of CMEIAS data analysis (e.g., community ecological succession), see Liu et al. 2001 Microbial Ecology 41: 173-194, and the Publications using CMEIAS page at the CMEIAS website. Also, the CMEIAS v. 1.27 download contains a training tutorial (text in Appendix 1) operated directly in ImageTool, plus an accompanying worksheet pdf file to follow the generation of typical data, graphics, and computations of community analysis.

Fig. 32. Percentage of total cell abundance in communities A and B represented by each size (area) class (32A) and morphotype (32B).
Table 2. Community morphological diversity indices

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Community A</th>
<th>Community B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphotype Richness =</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Total cell abundance =</td>
<td>170</td>
<td>170</td>
</tr>
<tr>
<td>Simpson Dominance (I) =</td>
<td>0.641</td>
<td>0.241</td>
</tr>
<tr>
<td>Simpson Diversity (D) =</td>
<td>0.359</td>
<td>0.759</td>
</tr>
<tr>
<td>Max of D =</td>
<td>0.805</td>
<td>0.914</td>
</tr>
<tr>
<td>Simpson evenness =</td>
<td>0.446</td>
<td>0.830</td>
</tr>
<tr>
<td>Inverse dominance (d) =</td>
<td>1.560</td>
<td>4.152</td>
</tr>
<tr>
<td>Max of d =</td>
<td>5.121</td>
<td>11.692</td>
</tr>
<tr>
<td>d evenness =</td>
<td>0.305</td>
<td>0.355</td>
</tr>
<tr>
<td>Log used for H' =</td>
<td>2.718</td>
<td>2.718</td>
</tr>
<tr>
<td>Shannon Diversity (H') =</td>
<td>0.773</td>
<td>1.726</td>
</tr>
<tr>
<td>H' max =</td>
<td>1.609</td>
<td>2.398</td>
</tr>
<tr>
<td>H' evenness(J%) =</td>
<td>0.480</td>
<td>0.720</td>
</tr>
<tr>
<td>Brillouin diversity =</td>
<td>0.728</td>
<td>1.622</td>
</tr>
<tr>
<td>Brillouin max =</td>
<td>1.551</td>
<td>2.351</td>
</tr>
<tr>
<td>Brillouin evenness =</td>
<td>0.469</td>
<td>0.690</td>
</tr>
<tr>
<td>Hill's N1 =</td>
<td>2.167</td>
<td>5.618</td>
</tr>
<tr>
<td>Hill's N2 =</td>
<td>1.560</td>
<td>4.152</td>
</tr>
<tr>
<td>Hill's evenness =</td>
<td>0.720</td>
<td>0.739</td>
</tr>
</tbody>
</table>

Comments on the parameters reported in Tables 2 and 3:
All parameters are computed in EcoStat (Trinity Software) from inputs of CMEIAS data on the number of different morphotypes (substitute for species) found and the distribution of abundance among each of them. The indices of community characteristics include measures of morphotype richness, diversity, dominance, and evenness. Community similarity analyses compare the similarity and dissimilarity of morphological diversity among the two communities. Some parameters are significantly influenced by sample size, and the two communities analyzed each contain equal (170 cells) sample sizes. Consult pg 15-16 of Howard Towner's *ecostat.pdf* manual for mathematical details.

Table 3. Community similarity analyses of morphological diversity in Communities A & B

<table>
<thead>
<tr>
<th>Prop. similarity (%) =</th>
<th>48.824</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jaccard coefficient =</td>
<td>0.455</td>
</tr>
<tr>
<td>Sorensen coefficient =</td>
<td>0.625</td>
</tr>
<tr>
<td>Morisita index =</td>
<td>0.634</td>
</tr>
<tr>
<td>Horn index =</td>
<td>0.758</td>
</tr>
<tr>
<td>Sneath/Sokal distance =</td>
<td>0.663</td>
</tr>
<tr>
<td>Bray/Curtis distance =</td>
<td>0.512</td>
</tr>
<tr>
<td>Chord distance =</td>
<td>0.769</td>
</tr>
</tbody>
</table>

CMEIAG / EcoStat analyses indicate that communities A and B have **48.82%** proportional similarity in morphological diversity, with Community B being ~ **2.2-fold** higher in morphotype richness and diversity indices and ~ **1.5-fold** higher in distribution of morphotype evenness.

These features illustrate how CMEIAS can strengthen the microscopy-based approaches that compliment other methods (e.g., 16S rDNA-based, nutritional versatility, FAME, etc) of polyphasic analysis to characterize the structure and function of complex, actively growing microbial communities *in situ* without cultivation. **Final reminder:** images to be analyzed in CMEIAS must be of high quality and 8-bit grayscale, and each foreground object of interest must be segmented, at least 30 pixels in size, and have a brightness range allowing it to be segmented from all background pixels using the thresholding routine in ImageTool. **Enjoy CMEIAS!☺**

Frank B. Dazzo, Michigan State Univ. December, 2003
Welcome to this hands-on CMEIAS v. 1.27 Training Tutorial, which is designed to accelerate your learning of the major important features offered in CMEIAS/ImageTool v.1.27 used in the image analysis of microbes. The macro was prepared by Frank Dazzo, Hassan Hammoud and Jose Zurdo at Michigan State University, East Lansing, MI 48824 USA. For questions or comments on this tutorial macro, email F. Dazzo at cmeiasfbd@msu.edu.

Every time this macro advances to the next dialog box, it will automatically run the *.wav sound file linked to your Windows "Asterisk" function (Start > Settings > ControlPanel > Sounds > Events > Windows > Asterisk). You may prefer to unlink any wav file to the Windows Asterisk sound or install one that you would enjoy hearing multiple times during this training session. "Utopia Asterisk.wav" is quite pleasant and available at <http://www.salaam.demon.co.uk/Sound/>.

This macro only allows you to proceed forward. No option exists to "undo", go back to revisit earlier steps, or minimize ImageTool while running this macro. You can click the Escape key repeatedly to skip steps without performing selected tasks and/or quickly reach the end to close this tutorial macro.

It is assumed that you are already familiar with the operation of the host program, UTHSCSA ImageTool ver. 1.27. ImageTool provides an operator manual, Help files, and an object analysis tutorial in its program download to help you learn its operation. The Results window should already be open, sized to display the first 4 columns by 18 rows, and moved to the far right side of the ImageTool workspace. Maintain this open status of the Results window throughout this tutorial.

This CMEIAS v. 1.27 tutorial will describe how to: (1) select the required preference settings, (2) perform an automatic object analysis of cells in a community image, (3) optimize the upper class...
limits of bins to perform an IT/CMEIAS-1 classification of the morphotype diversity in a simple microbial community, (4) compare the cell size distribution of different complex communities using the IT/CMEIAS-1 classifier, (5) measure the morphological diversity of complex microbial communities using the CMEIAS-2 morphotype classifier, (6) edit morphotype classification results directly on the image so that the final output of morphotype classification data fully conform to the users' specifications, and (7) illustrate how image analysis data generated by CMEIAS can be used to compute numerous indices that compare the morphological diversity, dominance, evenness, and similarity of complex microbial communities.

pause

You should have 2 documents readily available (either hard-copies or opened and minimized) while running this training tutorial. These are the CMEIAS v.1.27 operator manual (Cmeias127.pdf), and the CMEIAS v. 1.27 tutorial worksheet (CmeiasTutorialWorksheet.pdf).

Pause

The CMEIAS 1.27 tutorial worksheet contains 9 tables and 10 figures of data generated by this recorded macro and is provided to enhance the efficiency of this training tutorial plus show various ways to illustrate CMEIAS/ImageTool image analysis data. Take a minute to briefly view the various figures and tables in the worksheet now.

pause

You will analyze 3 different microbial community images in this tutorial. These are in the CmeiasHelp folder downloaded in the Cmeias127.zip file and should be copied to your Uthscsa/ImageTool/Help folder. Each image is an 8-bit binary Tiff containing 170 microbes, representing the distribution of size and abundance among different bacterial populations in methanogenic bioreactor communities. Small snapshots of these 3 images are combined into Fig. 1 in the tutorial worksheet.

Pause

Now we will set the various preferences to perform CMEIAS object analysis and classification. While making these settings, please consult the CMEIAS 1.27 operator manual for details describing what each selection does and why it's necessary for CMEIAS image analysis.

Pause

The Find Objects tab (Setting > Preferences > Find Objects) has settings that relate to the process of finding the foreground objects using the ImageTool threshold procedure. For most CMEIAS automated object analyses and morphotype classifications, select the "Search in AOI", select the "Automatically Select Objects", check "Exclude background", specify 498 for the "Maximum # of Objects" and "Set minimum size (pixels)" of foreground objects to "30" (a screenshot of these settings is displayed on the next page). Click OK here to display the various preference pages, select the "Find Objects" tab, enter these settings, and then select "Apply" and "OK" to activate them for this tutorial.

command
preferences
Next under the Display options in the "Find Objects" tab, select "Show object count in a message box", "Show object numbers on original image", and "Show object outlines on original image". Then double-click the "Choose Font" and "Choose Color" rectangular boxes to specify your preferred choices. We recommend that these image annotations be set as a blue 14 pt. Tahoma font and a bright magenta color (Color palette/2nd row/last cell) to define the object contour. Click OK to return to the preference window, then make these selections in the Find Objects page, followed by "Apply" and "OK".

Next, click OK to open the Settings > Preference > Image tab and set the initial zoom ratio to 1:2 for this tutorial.

- pause

command preferences

- pause

command preferences

- pause
Now, open the "Measurement Feature" tab (settings > preferences > measurement features) to specify the CMEIAS/ImageTool measurement attributes to be extracted from each microbe in the 1st object analysis. Check the boxes for the cell Length, Width, and Width/Length ratio. The latter is a shape measurement feature located within the shaded frame labeled "CMEIAS Morphotype Classifier"; not to be confused with the Length/Width ratio. Also for all analyses in this tutorial, set the Measurement Precision to 2 decimal places. Click OK to return to the Measurement Feature preference window and introduce these settings.

Command
preferences

![Measurement Features Window](image)

Pause
Click OK to open the CoccusRegularRodFilament.tif image.

open
C:\program files\Uthscsa\ImageTool\Help\CoccusRegularRodFilament.tif

pause
Spatial calibration of an image is a very important step preceding the measurement of object sizes, so that the output values are reported in user-selected units rather than the pixel default. This interactive step is best done while zoomed in to magnify the ends of the bar scale line. This is normally done manually by clicking the "zoom plus" or "zoom minus" shortcut icons (+ or - next to the magnifying lens) in the toolbar. In the next steps you will spatially calibrate the image for object analysis using the 10 um bar scale located in its lower right corner. Click OK to zoom in on the image.
Follow these steps to spatially calibrate the image. First, move the image so its upper left corner lies at the upper left corner of the ImageTool workspace. Then grab the lower right corner of the image and enlarge it diagonally a couple of inches, and scroll to display all of the bar scale. Next, with great care -- position the tip of the pencil cursor exactly on the left edge of the bar and click once, then position the cursor on the right edge of the bar and double-click to create a line of exactly the same length. After completing this step, a window will display, asking "How long is the line?" Check the radio button for "microns" (= micrometers) and enter a Length of 10.00 in the white window.

The next step is to find the foreground objects of interest to be analyzed in the segmented image using the ImageTool brightness thresholding procedure (Analysis > Object Analysis > Find Objects).

In the next step, a box will display asking you to select the threshold method to find the foreground objects of interest. For this example, select "None > OK" since the image contains only black and white pixels (= binary). Then use the mouse cursor to draw a blue line polygon on the image (click once at each corner) to enclose all foreground objects while excluding the bar scale in the lower right corner. To close the polygon, double click the last corner. Once completed, each foreground object found within the polygon will automatically become surrounded by a colored line and numbered consecutively from the bottom-up.
Click OK to perform an object analysis on the foreground microbes found in the image.

C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objanal.dll

Pause

Take a look at the layout of your object analysis data reported in the ImageTool Results window. It lists rows of objects found (each numbered as in the thresholded image) and columns of values for each measurement attribute extracted from the corresponding objects. Normally at this stage you would copy these object analysis data to your Windows spreadsheet program. That's not necessary here since they are already copied to Table 1 of the tutorial worksheet. View the top portion of that table now in the pdf worksheet.

Pause

Next, scroll the tutorial worksheet to view Fig. 2, which is a 2D scatter plot of Cell Length vs. Width in this dataset. Note the locations of clustered objects and outliers.

Pause

Now scroll the tutorial worksheet to view Fig. 3 which is a line plot of the fifth column of Table 1: an ascending sort of each cell's Width/Length ratio plus various grouped labels (text, horizontal lines, arrows) inserted. Then return back to the macro.

Pause

Note in Fig. 3 the two data points next to small black arrows where obvious breaks occur. When these data are plotted in your spreadsheet program, you can position the cursor over them to display a text box indicating their corresponding X and Y axis positions in the plot (the Y value is their computed width/length ratio). Based on that information, two horizontal lines have been introduced to indicate the optimized upper class borders of 0.5 and 0.0625 W/L that will efficiently separate the three different microbial morphotypes present in this community image.

Pause

Our next step is to perform an IT/CMEIAS-1 object classification of this image using these 2 optimized upper class borders of the cell W/L attribute. These bin limits will satisfy the requirements needed to classify each microbe in this image into its corresponding morphotype (coccus, regular rod, or unbranched filament).

Pause

In the Settings > Preferences > Object Classification tab, select the "Report on classifications using a single measurement feature", "Value Range", "Number of objects in class", and "Display new images showing objects colored by classification". Deselect the "Mean" and "Standard Deviation" values in all classes.
Next, input the parameters for the IT/CMEIAS-1 classification of this image. Pull down the "Attribute" box and select the Width/Length feature. Then click the Load button and select the OptimizedWidthLength.ocd file in the ImageTool\Calibration folder. This will automatically enter the upper border limits (0.0625 for the 1st, 0.5 for the 2nd) in the top two white boxes. This scheme will sort the microbes in this image into 3 bin classes; the 1st group has a W/L of 0.0625 or less (unbranched filaments), the 2nd has a W/L between >0.0625 and 0.5 (regular rods), and the 3rd has a W/L of > 0.05 (cocci). When you click "OK", the IT/CMEIAS-1 object classifier will run and report the data in the Results window.

plug-in
C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objclass.dll

Your new classification image should look like Fig. 4 in the worksheet. Notice that each microbial cell has been pseudocolored according to the IT/CMEIAS-1 width/length class to which it has been assigned (red - class 1, green - class 2, blue class 3). The object counts in each class displayed in the Results window should match Table 2 in the worksheet.

Our next task is to perform a CMEIAS comparative analysis of the microbes in the Community-A and Community-B images, illustrated in Fig. 1 of the tutorial worksheet. We will first analyze their cell size distribution using object area measurements and the IT/CMEIAS-1 object classifier. Click OK to open the Community-A.tif image.

open
C:\program files\Uthscsa\ImageTool\Help\Community-a.tif
Next do a spatial calibration using the 10 um bar scale on this image using the same steps described earlier. Click OK to perform the Spatial Calibration.

```
command
zoom in
command
zoom in
command
spatial calibrate
command
zoom out
command
zoom out
```

Now find the foreground objects using the same threshold steps described earlier, making sure to exclude the bar scale from the AOI polygon.

```
command
find objects
```

Click OK to open the Preferences window, select the Measurement Features tab, and check the Area attribute for object analysis (deselect all other choices).

```
command
preferences
```

Now perform the object analysis of cell areas.

```
plug-in
C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objanal.dll
```

Next, open the Community-B.tif image, spatially calibrate it using the 10-micrometer bar scale, and perform the threshold routine to find the foreground microbes of interest.

```
open
C:\program files\Uthscsa\ImageTool\Help\Community-b.tif
```

command
zoom in
command
    zoom in
command
    spatial calibrate
command
    zoom out
command
    zoom out
command
    find objects
pause
Click OK to perform the object analysis on this image. Note that the reporting of data extracted from microbes in this image will be CONCATENATED in the Results window below the previous data extracted from the Community-A.tif image.

plug-in
    C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objanal.dll

Pause
Open the tutorial worksheet and view the top portion of Table 3 containing the cell area object analysis data for both images, plus 2 additional column arrays showing the same data after an ascending sort.

Pause
Figure 5 in the worksheet is a line plot of the sorted object areas in communities A and B. Although this straightforward object analysis produces accurate data, the results reveal only marginal differences in their cell size distribution. A more powerful approach to analyze this community characteristic would be to optimize the class bin limits in regions where the 2 communities differ most in Fig. 5, and then apply these values to a IT/CMEIAS-1 classification of cell areas. Let's do that analysis to emphasize this point.

Pause
For this object classification, we do not need to produce a new image with the objects colored according to their classification, so open the Object Classification preference page and deselect this feature.

command
    preferences
Pause
Open, spatially calibrate, find objects and perform an IT/CMEIAS-1 classification of the cell area distribution in the Community-A.tif image.

```
open  
C:\program files\Uthscsa\ImageTool\Help\Community-a.tif
command  
zoom in
command  
zoom in
command  
zoom in
command  
spatial calibrate
command  
zoom out
command  
zoom out
command  
find objects

Pause
For your convenience, we have already created a calibration file of the optimized area bin increments for use in this next IT/CMEIAS-1 object area classification. Click OK to display the window to set preferences for the IT/CMEIAS-1 classifier, select "Area" as the measurement attribute, click the Load button and select the file "OptimizedAreaBins.ocd" to introduce these prerecorded bin increments. Then click OK to perform the object classification.
```

```
plug-in  
C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objclass.dll
pause
Now repeat these steps to perform an identical classification of cell areas using the same optimized upper class borders for the Community-B image.

open  
C:\program files\Uthscsa\ImageTool\Help\Community-b.tif
command  
zoom in
command  
zoom in
command  
```
spatial calibrate

command
zoom out

command
zoom out

command
find objects

plug-in
C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objclass.dll

pause
The data on classification of object areas for these two community images are combined in Table 4, and the corresponding results are plotted as vertical stacked and vertical clustered bar graphs in Figs. 6A and 6B of the tutorial worksheet. This illustrates another way that CMEIAS/IT can extract quantitative data indicating the similarities and differences in distribution of microbial abundance within different communities.

Pause
Now we are ready to introduce the CMEIAS-2 morphotype classifier, which is the most innovative and powerful feature of CMEIAS v. 1.27. This object classifier uses various pattern recognition algorithms optimized for 11 major microbial morphotypes represented by 98% of the genera described in the 9th Edition of Bergey's Manual of Determinative Bacteriology. Scroll to Fig. 7 in the tutorial worksheet to view the characteristics of these 11 morphotypes classified by CMEIAS, including each one's specific pseudocolor assignment.

pause
In order for CMEIAS to perform these automated morphological classifications, we must first measure all required shape attributes of the microbes in an object analysis of the image. These measurement features are defined mathematically in the CMEIAS Operator Manual. Click OK to open the preference window, select the Measurement Feature tab, click all 7 shape measurement features located in the left framed box labeled "CMEIAS Morphotype Classifier", and deselect all other measurement feature choices. Click OK to make these selections now.

pause
Next, we must instruct the program to perform the CMEIAS-2 object classification. In the Settings > Preferences > Object Classification page, select "Report on CMEIAS Morphotype
Classifier using multiple measurement features" and "Display new image showing objects colored by classification", and deselect the other choices on this page. It is most important that we include the classification result image for the CMEIAS-2 object classification since it serves as the way to inspect the classification results and correct any errors if found. Click OK to make these selections.

command preferences

Pause

Now let's open the Community-A.tif image, find the foreground objects, and perform the object analysis of cell shapes. Spatial calibration of the image is not necessary here since all shape attributes are unitless measurements. Click OK to do these steps.

open
C:\program files\Uthscsa\ImageTool\Help\Community-a.tif

command
find objects

plug-in
C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objanal.dll

Pause

Maximize the tutorial worksheet at this point to view the results of this object shape analysis (for the first 39 objects) in Table 5. Consult the CMEIAS Operator Manual for the abbreviations of shape measurement features labeled in each column.

Pause

ImageTool will always ask if you want to save the current object analysis data in the Results window because they will be overwritten when acquiring object classification data (and visa versa). The object analysis data of shape attributes required to perform a CMEIAS-2 morphotype
classification are usually not saved unless additional measurement features (e.g., cell area, length, etc) are also included. This is because these data are typically only used to compute the pattern recognition algorithms of the CMEIAS morphotype classifier.

Pause

Now click OK to perform the CMEIAS-2 morphotype classification.

plug-in

C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objclass.dll

pause

Voila! Notice in the new classification image (copied to Fig. 8 in the tutorial worksheet) how each object is pseudocolored according to its specific CMEIAS morphotype, as detailed in the outline of Fig. 7. The classification report displayed in the Results window indicates the microbial count in each morphotype class in this image, using text with the same pseudocolors as illustrated in Fig. 7 and the image itself, and a useful, sum total row. Although the Results window always displays the mean and standard deviation in the CMEIAS-2 morphotype classification, they are really not useful in this particular image analysis.

Pause

Next, open image Community-B.tif and perform the same find objects/object analysis/object classification tasks.

open

C:\program files\Uthscsa\ImageTool\Help\Community-b.tif

command
find objects

plug-in

C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objanal.dll

plug-in

C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objclass.dll

Pause

Witness the awesome computing power of CMEIAS. Spend a minute to examine this classification result image and note how CMEIAS pseudocolors each object according to its morphotype classification. Maximize the tutorial worksheet and scroll up to revisit Fig. 7 again to relate these pseudocolor assignments to the other characteristics that distinguish each morphotype.

Pause

Extensive testing indicates that CMEIAS classifies microbial morphotypes in properly edited images with an overall accuracy of 97%. The major source of this 3% error rate is the existence of cells whose morphology lies within the real-world continuum that overlaps the assigned borders of closely related microbial morphotypes that are defined in 14-dimensional space by the shape recognition algorithms in the CMEIAS program. Consult our Liu et al. 2001 Microbial Ecology
article on CMEIAS and the CMEIAS 1.27 Operator Manual for a complete discussion of the sources of classification error and how CMEIAS is designed to minimize and address them.

Pause

We will now illustrate this type of error and how to correct it in a CMEIAS-2 morphotype classification using the Community-B.tif image.

Pause

Find the short, thick red cell near the center of the classification result image (a white arrow points to it in the worksheet Fig. 9A). CMEIAS classified this coccobacillus as a coccus rather than a regular rod because of its high roundness and circularity shape values, and its length is less than twice its width. However, some CMEIAS users may prefer to classify it as a regular rod instead. CMEIAS was designed to facilitate the users' desire to edit such borderline object classifications within the pseudocolored image and revise the object classification data accordingly.

Pause

Activate this edit feature by selecting Analysis > Object Analysis > Edit Classification Results. This displays a "Reassign Category Label" selection box containing radio buttons next to all morphotypes in the CMEIAS-2 classifier. Next, move the (magic wand) cursor over the pseudocolored object you wish to edit, and click it once (the selected object will flash on and off) to indicate its assigned morphotype (here the radio button for coccus will be filled) (see Fig. 9B). Then, select the radio button corresponding to the new classification assignment for that object (here select the Regular Rod which will recolor the selected object as true blue, see Fig. 9C). Finally, select the Done button after all edit reclassifications are made to update the classification data in the Results window. Click OK now to automatically open the edit selection box and perform this edit morphotype classification routine.
Table 6 indicates the object classification data containing the cell counts for each morphotype found in both community images. Note that we have included 2 columns of community B (before and after editing) and have highlighted how the classification data have been changed. The regular rod count increased by 1, and the coccus count correspondingly decreased by 1 in the edited results.

Pause

Now view how these morphotype classification data are displayed in the tutorial worksheet. Table 7 is a descending sort of the Table 6 results. Figs. 10A and 10B are vertical clustered and vertical stacked bar graphs that indicate the total and relative abundance of each morphotype in the 2 community images, respectively, clearly illustrating differences between these 2 microbial communities.

Pause

These classification data can be used to compute various indices that quantify the similarities and differences in morphological diversity between 2 or more microbial communities. Table 8 and 9 present the computed results and their interpretation. Gain a sense of appreciation how CMEIAS image analysis provides several new opportunities to strengthen microscopy-based approaches for understanding microbial ecology.

Pause

This ends the Cmeias 1.27 training tutorial. Enjoy CMEIAS!
CMEIAS Object Analysis Macro
(CmeiasObjectAnalysis.itm)

Use this macro to guide you through the steps to perform an object analysis on your image.

Pause
This CMEIAS macro is provided to help you perform an object analysis on your images. If you want to view your data while they're being extracted (optional, recommended) but your Results window is currently minimized, quickly cycle to the end of this macro with multiple clicks of the Escape key and then maximize it. To proceed, open the first image you wish to analyze.

Command
open

Pause
In the Find Objects tab, select the parameters you want ImageTool to use to find your microbes of interest.

Command
preferences

Pause
In the Measurement Features tab, select those attributes you'd like to extract from the microbes in the image. Also indicate the precision (decimal places) to report your data.

Command
preferences

Pause
Next perform a spatial calibration on your image.

Command
spatial calibrate
Pause
Now, select either manual or automatic thresholding from the dialog box and perform the
brightness threshold procedure to find your foreground objects of interest.

command
find objects

pause
Now perform the object analysis on the thresholded image, and view the data in the Results
window. You may clear, cut, or copy these data to the Windows clipboard using the Edit main menu.

plug-in
C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objanal.dll
This CMEIAS macro is provided to help you perform an IT/CMEIAS-1 classification of objects in your image using any single measurement attribute (except Fourier Descriptors) featured in CMEIAS/ImageTool v1.27. If you want to view your data while they're being extracted (optional, recommended) but your Results window is currently minimized, quickly cycle to the end of this macro with multiple clicks of the Escape key and then maximize it. To proceed, open the first image containing the microbes you wish to classify.

command
open

Pause

In the Find Objects tab, select the parameters you want ImageTool to use to find your microbes of interest.

command
preferences

pause

In the Object Classification tab, select "Report on classifications using a single measurement feature" (= IT/CMEIAS-1 classifier), and the associated parameters to report on this classification. Typically these will include the Value Range and Number of Objects; optionally also include the Mean and Std. Dev. of all objects in the classification. Also select if you want ImageTool to display a new image showing objects pseudocolored by their classification.

command
preferences

Pause
Now, perform a spatial calibration of your image if you are classifying the objects using a size attribute. If classifying by shape or grayscale level (e.g., width/length, mode gray level), spatial calibration of the image is not necessary. If the latter case, draw any line on the image and accept its default dimension in pixels.

command
spatial calibrate

Pause

Next, select either manual or automatic thresholding from the dialog box and perform the brightness threshold procedure to find your foreground objects of interest.

command
find objects

pause

Now perform the object classification on the thresholded image. Select the measurement attribute from the pulldown box and then enter the upper class limit of each bin in the object classification.

plug-in
C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objclass.dll

pause

Your object classification data should now be displayed in the Results window. You may cut or copy these data to the Windows clipboard, or clear the results using the corresponding selections from the Edit main menu.
Use this CMEIAS macro to perform a CMEIAS morphotype classification of microbes in your image using the multiple shape measurement attributes featured in CMEIAS/ImageTool v1.27. Before starting however, you must have no data from a previous analysis in the Results window. Click OK to continue, or the Escape button multiple times to end this macro and remove your data from the Results window.

Open the image containing the microbes you wish to classify.

In the Find Objects tab, select the parameters you want ImageTool to use to find your microbes of interest.

In the Measurement Features tab, select all 7 shape measurement attributes within the framed box labeled "CMEIAS Morphotype Classifier."

- XX -
In the Object Classification tab, select "Report on CMEIAS Morphotype Classifier using multiple measurement features." Also select if you want ImageTool to display a new image showing objects pseudocolored by their classification.

command
preferences

Pause

Next, select the type of brightness thresholding procedure (commonly manual) from the dialog box and use it to find your foreground objects of interest.

command
find objects

pause

Now the object analysis routine will extract the shape attributes required to classify the microbial morphotypes in the image.

plug-in
C:\Program Files\UTHSCSA\ImageTool\Plug-Ins\objanal.dll

pause

When ImageTool asks if you want to save the current contents of the Results window, answer "No." CMEIAS will then perform a morphotype classification of the microbes analyzed in the image and report the data in the Results window.

plug-in
C:\Program Files\Uthscsa\ImageTool\Plug-Ins\objcclass.dll

pause

Check the accuracy of the classification by inspecting the new image containing each microbe pseudocolored according to its assigned morphotype. If satisfied with the results, you may clear, cut, or copy the data from the Results window to the Windows clipboard using the Edit main menu. If the classification needs correction, open the reassign classification label routine (Analysis>Object Analysis>Edit Classification Results) and update the classification result before copying the data to the clipboard (see Cmeias Operator Manual for details).
Known problems with Uthscsa ImageTool 1.27 and CMEIAS 1.27:

1. The ImageTool 1.27 Results window accurately reports only on the first 498 objects in the image, regardless of how many objects are found (see sections 4.1.4, 4.2.2.6, and 6.6).
2. The Results window reports the mean and std. dev. for all values in a column data array (see sections 4.1.4 and 6.6). These are useful and correct statistics for object analysis data, but incorrect for the two object classification data since the 0 and TOTAL values are included in their computation.
3. The pseudocolors used to illustrate each object's classification are distinctively optimized for the CMEIAS-2 morphotype classifier but not for the IT/CMEIAS-1 object classifier in ver. 1.27.
4. The word "Micron" in the image spatial calibration routine in ImageTool 1.27 is the "micrometer" unit ($10^{-6}$ meter) and is replaced with the word "Micrometer" in ImageTool ver. 3.0.
5. Often ImageTool crashes while thresholding very large images containing many objects.
6. None of the 4 CMEIAS macros work properly using the Windows ’98 or XP operating systems. The errors occur when the macros try to activate the plugins automatically. The macros work fine when run in computers using Windows NT 4.0 or 2000. If necessary, you can still make use of the Cmeias127 Training Tutorial by following the text provided in Appendix I and manually performing the various object analyses and classification steps using the same images, calibration and pdf files provided in the Cmeias127.zip download.
7. The objclass.dll plugin does not perform properly in Windows 98.

Most of these problems are/will be resolved in ImageTool ver. 3.0 and CMEIAS ver. 3.0 currently under development. Let us know via the Join Our Mailing List page in the CMEIAS website <http://cme.msu.edu/cmeias/> of any other problems you encounter using CMEIAS 1.27 in Uthscsa ImageTool 1.27 so we can address them in CMEIAS ver. 3.0.

Enjoy CMEIAS!

Frank Dazzo
Michigan State University