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Comparing 2-D Electrophoretic Gels Across Internet Databases

An Open Source Application

Peter F. Lemkin, Gregory C. Thornwall, and Jai Evans

1. Introduction

In ***(1–4)*** and in the previous edition of this book ***(5)***, we described a Web-based computer-assisted visual method called Flicker for comparing two-dimensional (2-D) protein gel images across the Internet using a Java applet. We originally used the flicker method in the GELLAB 2-D gel analysis system ***(6–9)***. The applet (primarily for Web-based analysis using a Web browser) has since been converted to a Java standalone application that can run on a user's computer. The new application also can access images from the Web but can now more easily access the user's data on their local computer. The new version is available as open source on http://open2dprot.sourceforge.net/Flicker, where the executable program and the source code are available. Some of the code was derived from the old Flicker applet program and some from the MicroArray Explorer ***(10)***—an open-source data-mining tool for microarray analysis (http://maexplorer.sourceforge.net/).

Because Flicker now can run on a user's computer, this gives them the ability to perform real-time comparisons of 2-D-gel image data with gel images residing on remote Internet databases on the Internet, or on the user's local file system, or a combination of both sources.

This approach may be useful for comparing similar protein samples created in different laboratories to help putatively identify or suggest possible protein spot identification. The gels should be run under similar pH and molecular-weight ranges if possible. Although available for over three decades, 2-D polyacrylamide gel electrophoresis (2-D PAGE) is still routinely used ***(11)***, even considering the now common use of mass spectrometry ***(12–17)*** and recently protein arrays ***(18)*** for protein identification.

Recent advances, such as isoelectric focusing (IEF) "zoom" fractionation gels ***(19)*** that divide the protein sample by pH range or immunoaffinity subtraction with liquid chromatography (LC) ***(15)***, greatly increase the resolution and numbers of spots able to be discriminated by subsequent 2-D gel electrophoresis. Another increasingly common image comparison technique uses two to six cyanine dyes using dye multiplexing to label multiple control and experimental samples run in the same gel, such as Amersham's differential in-gel electrophoresis (DIGE) ***(20)***, and scanned with systems

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like Perkin Elmer's ProExpress ***(21)***. Multiple scans of the same gel using different color filters can then be color mapped to see the contributions of the different samples. This is useful if one has control over the experimental design when determining the reference gel, set of control gels, and experimental gels. However, it does not solve the problem of trying to putatively compare one's own sample against an Internet reference gel where they have identified protein spots.

A number of 2-D-gel databases that contain gel images are available on the Web for various types of tissues. Proteins are identified for some of the spots in a subset of these databases. Both WORLD-2DPAGE and 2D Hunt on the http://www.expasy.org/ server can be used to find Web URL addresses for a number of 2-D protein gel databases. Google searches are also used, and we link to these sites in the Help menu. Many of these databases contain 2-D-gel images with identified proteins. Some of these databases let you identify spots in their gels by clicking on a spot in their gel image shown in your Web browser. It then queries their Web server database to determine whether the spot you pointed to is in that database and report its identity if found. These "clickable" 2-D-gel map images are often published using a common federated database (DB) schema ***(22–23)***. One of the more interesting databases is SWISS-2DPAGE ***(22–25)***, accessible from the Expasy site. It has a large number of tissues with over 30 gel databases, including a wide range of human tissues, mouse, *Escherichia coli*, *aribidopsis*, *dictyostelium*, and yeast. Their site also has a series of IEF zoom fractionated gels for *E. coli*.

We have incorporated links to these SWISS-2DPAGE database gel-map images so they may be loaded and accessed directly from Flicker after having putatively matched a spot in your gel with one in the SWISS-2DPAGE gels. If you have loaded one of these active gel-map images in Flicker and enabled the database access, then clicking on a spot in that image will pop up a Web page as it tries to look up the spot in the SWISS-2DPAGE database. If a SWISS-2DPAGE data entry exists for the spot coordinates you have selected, then it will report the corresponding protein; if not, it will tell you it can't be found. Access to PIR UniProt, iProClass, and iProLink (http://pir.georgetown.edu) is also available.

By comparing one's own experimental 2-D-gel image data with gel images of similar biological material from these Internet reference databases, it may be possibile to use the spots in these reference gels to suggest the putative identification of apparently corresponding spots in your gels. The image analysis method described here allows scientists to more easily collaborate and compare their gel image data over the Web.

1.1. How Can We Compare Two Gels?

When two 2-D gels are to be compared, simple techniques may not suffice. There are several methods for comparing two gel images: (1) put the images side by side and visually compare them; or (2) slide one gel (autoradiograph or stained gel) over the other while back lighted; or (3) build a 2-D-gel quantitative computer database from both gels after scanning and quantitatively analyzing these gels using a 2-D-gel database system; (4) more recently, dye multiplexing ***(20)*** has been used to label different samples in the same gel. A variant of the latter method is to spatially warp two gels to the same geometry and then pseudocolor them differentially. These methods may be impractical for many investigators, since in the first case the physical gel or autoradio-

graph from another lab may not be locally available. The first method may work for very similar gels with only a few differences. The second method will work better for gels that are not so similar but that have local regions that are similar. The third method may be excessive if only a single visual comparison is needed, because of the costs (labor and equipment) of building a multi-gel database solely to answer the question of whether one spot is probably the same spot in the two gels. The fourth method may also not be practical if you want to compare your sample against an existing reference gel.

1.2. The Flicker Program

We describe a computer-based image comparison technique called Flicker that has been used for years in finding differences in star maps in astronomy.

The Flicker program runs on most computers. It is started as one would any program after it is downloaded from the Flicker server and installed (*see* **Subheading 3., step 1**). One gel image may be read from any Internet 2-D-gel database (e.g., SWISS-2DPAGE), the other may reside on the investigator's Web server where they were scanned or copied, or the two gel images may be from either Web server source.

Figure 1 shows the Flicker application after it has been started with some demo gels. You interact with the program by clicking or dragging the mouse in the left or right images, adjust parameter scrollers (upper right), set interaction modes (checkboxes upper left), keyboard short-cut commands, and primarily pull-down menu commands.

1.3. Notation in This Paper

We use the notation <menu name> | <command> throughout this paper to indicate menu commands . The <menu name> indicates one of the pull-down menus: File, Edit, View, Landmark, Transform, Quantify, Help. The <command> indicates one of the commands in that menu. Table 1 summarizes the menu commands. Some of the commands have alternative keyboard shortcuts activated by using the Control key with another key, and are indicated as Control-<key> or C-<key> (e.g., C-A). The checkbox menu commands are indicated with a ☐ prefix. Checkbox commands may be toggled on and off.

The gels in the two lower left and right images are specified by the user with the Flicker Files menu. Gel images may be loaded from: the local computer **File | Open image file**, or any Internet site with GIF, JPEG, TIFF, or PPX images (with .gif, .jpg, .tiff or .tif, or .ppx GELLAB-II format ***(8)*** file extensions) using the **File | Open image URL** command. In addition, the installation provides a few demonstration images **File | Open demo images**, which loads pairs of comparable images. You may also specify active gel images from Web servers as described below.

Flicker is also capable of interacting with federated 2-D-gel databases to retrieve data on individual protein spots if one of the gels is a federated gel having an associated clickable gel-map database. After aligning gels in Flicker, you enable federated database access in Flicker and then click on a spot in the gel belonging to the federated database (*see* **Fig. 2**). This causes a Web page to pop up with information from the federated server describing that protein. We provide menu entries **File | Open active map image | …** to let you load one of the SWISS-2DPAGE gel images.

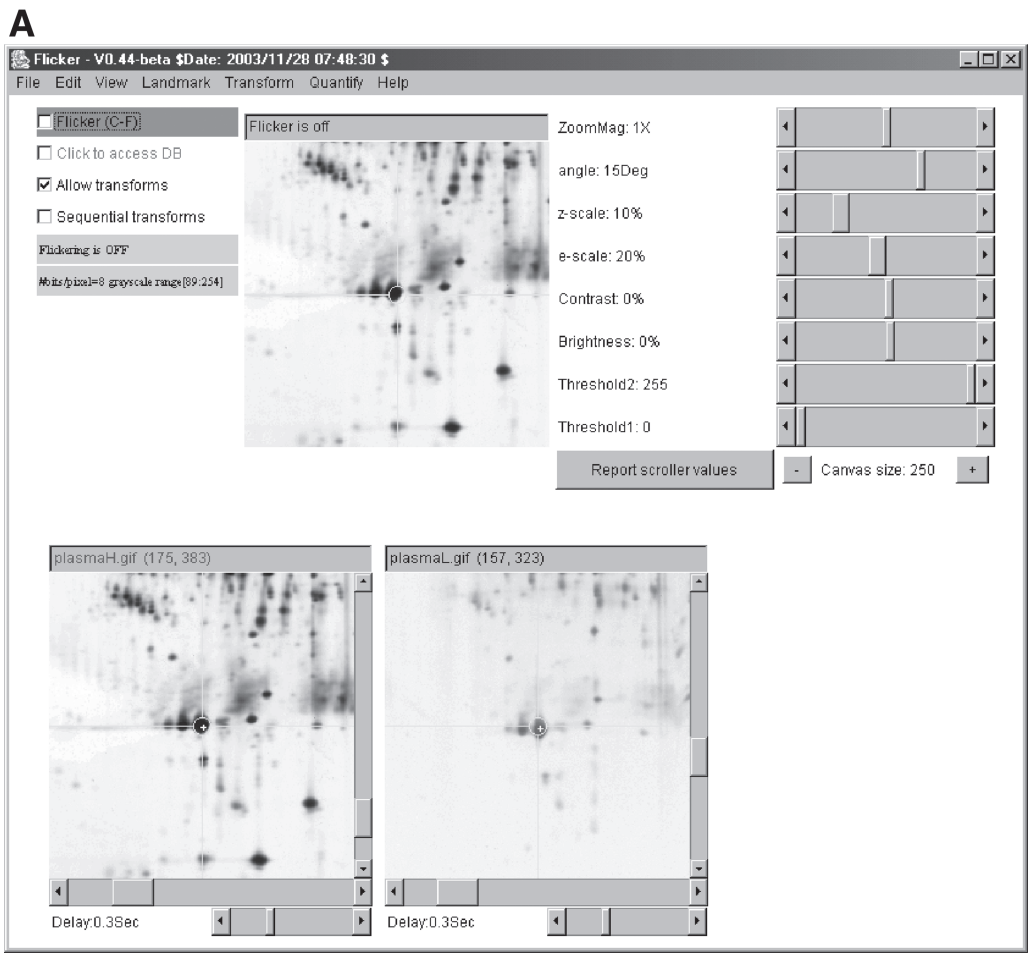

Fig. 1. Screen view of initial Flicker program. **(A)** shows the pull-down menus at the top used to invoke file operations, editing, view selection, landmarking, image transforms, spot quantification, and help. A set of scroll bars on the right determines various parameters used in some of the transforms. The "File" menu options include opening a new gel image. Checkboxes on the left activate flickering and active gel-map access if the data support it. A set of status lines appears below the checkboxes, indicating the state of operation and various other messages. The flicker image is in the upper middle of the frame when it is enabled. The two labeled human blood plasma gel images are shown in the bottom scrollable windows, which may be positioned to the region of interest. These windows also have associated flicker time-delays used when flickering. Image plasmaH is an immobilized pH gradient nonlinear gradient gel from SWISS-2DPAGE in Geneva, and plasmaL is a carrier-ampholyte linear gradient gel from the Merril Lab at NIMH. Transformed image results are shown in the same scrollable windows. The four checkboxes are: "Flicker (C-F)" to enable/disable flickering; "Click to access DB" enables/disables access to a Web server that is associated with a clickable image DB if it exists for the selected image; "Allow transforms" enables/disables image transforms; "Sequential transforms" enables/disables using the last image transform output as input for the next image transform. The parameters used in various transforms are adjusted directly by first selecting an image and then adjusting its values. You can pop up the scrollable report window using the "Report scroller values" button. These parameters are saved when you save the state. You can

B

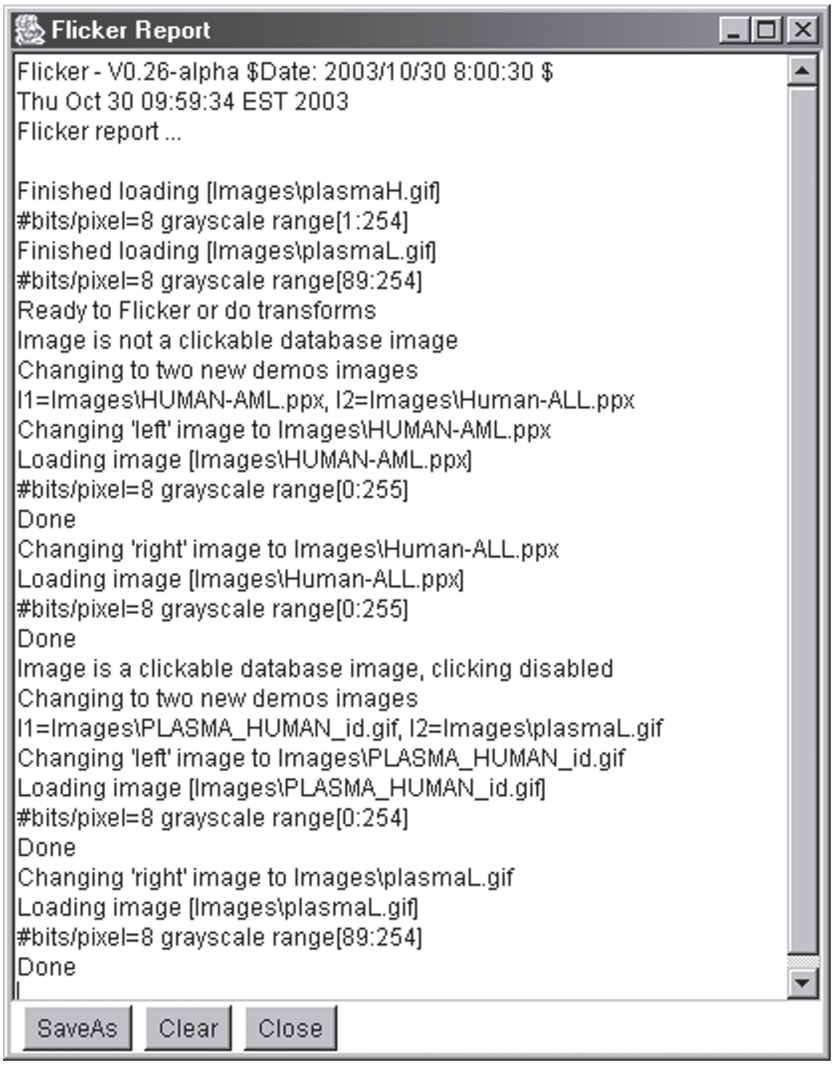

Fig. 1. (*continued*) change the size of the three image windows by using the "+" and "–" buttons. **(B)** The pop-up scrollable report window shows a log of all text output that appears in the status lines. It may be saved to a text file on the local disk.

You may load a gel image in the lower left or right image windows. First click on the image you want, to load the new image. Then select the active gel image you want using the entry **File | Open active map image | …** pull-down menu (e.g., select **… | SWISS-2DPAGE Human | Plasma**). Next, click on the other image and then use the other gel image you want to compare it with, using either **File | Open image file** or **File | Open image URL** commands.

The Flicker program is written in Java, a general purpose, object-oriented programming language developed by Sun Microsystems ***(26)*** http://java.sun.com/. Java has become a standard for portable Internet Web applications.

Table 1
Summary of Commands Available in Flicker Menu
Submenus are indicated by an underline and by the additon of the arrow symbol. Submenu items are indented. For brevity, not all submenus are shown; see Web site for all full documentation. Checkboxes are indicated by the "☐" prefix to the command. Shortcuts are indicated by the (C-<key>) at the end of the command. Commands are grouped by "----" in the menus (*see* Notes 5–7 and 9).

```
File menu - load images, load demo or user images, active map URLs, load/save
            Flicker .flk state, update program and data (from the Web server).
  Open image File - pop up gel image file browser
  Open image URL - pop up gel image URL dialog
  Open demo images ▶ - load pairs of demonstration gel images
  Open user images ▶ - load pairs of user's gel images from Images directory
     Pairs of images ▶ - directories with pairs of images in each user directory
     Single images ▶ - directories with single images in each user directory
     List user's images by directory - list the user's images
  Open active map image ▶ - load active gel image from the Internet Reference DBs
  Open recent images ▶ - load an image you have used recently
  Assign active image URL - to one of the open images to make it active
  ------
  Open state file - restore the Flicker state of previously saved session
  Save state file - save the Flicker state in current .flk state file
  SaveAs state file - save the Flicker state in new .flk state file
  ------
  Update ▶ - download and update your program and data from server
     Flicker program - to get the most recent release
     Active Web maps image DB - get latest active maps image database
     Demo Images DB - get latest demonstration images database
     Add user's Flicker Demo Images DB by URL - specify user demo data URL site
  ------
  Save Transformed image - of selected image as .gif file if transformed
  SaveAs Overlay image - the current overlay image
  Reset images - to the initial state when they were loaded
  Abort transform - abort any active image transforms
  ------
  Quit - exit the program, saving the .flk state of Flicker in the process

Edit menu - change various defaults.
  Canvas size ▶ - change the size of 3 image canvases and overall Flicker window
       Increase size (C-Numpad '+') - increase the canvas size
       Decrease size (C-Numpad '-') - decrease the canvas size
  Set colors ▶ - set default colors for the overlays
     Target colors ▶ - to change the target color
     Trial object colors ▶ - to change the trial object color
     Landmarks colors ▶ - to change the color of landmarks
     Measurement colors ▶ - to change the color of measurements
  ------
  Resize Flicker memory limits - set startup memory limit, 30Mb to 1784Mb.
  ☐ Use linear else log of TIFF files > 8-bits - take log of tiff data if > 8-bits
  ☐ Enable saving transformed images when do a 'Save(As) state'
  ☐ Use protein DB browser, else lookup ID and name on active images
  ------
  ☐ Auto measure, protein lookup in active server and Web page popup
  Select access to active DB server ▶ - select active server to use
     Use SWISS-2DPAGE DB access
     Use PIR UniProt DB access
     Use PIR iProClass DB access
     Use PIR iProLink DB access

  ------
  Reset default view - sets all view options to the defaults
  Clear all 'Recent' images entries - clears list of recently accessed images
```

(*continued*)

Table 1 (*Continued*)
Summary of Commands Available in Flicker Menu

View menu - change the display overlay options.
- Flicker images (C-F) - toggle flickering on and off
- Set view overlay options ▶ - enable/disable overlay view options
  - View landmarks - add landmarks to the overlay display in images
  - View target - add target to the overlay display images
  - View trial object - add trial object to the overlay display images
  - View Region Of Interest (ROI) - add ROI to the overlay display images
- Set view measurement options ▶ - enable/disable measurement view options
  - View measurement circle - add measurement circles to overlay display images
  - Use 'Circle'for measured spot locations - add circles, else '+'
  - Use '+' for measured spot locations - add '+', else circles.
  - Use 'spot number' for spot annotations - add spot number.
  - Use 'spot identifier' for spot annotations - add spot identifier
- Set gang options ▶ - enable/disable ganged images view options
  - Multiple popups - make multiple popup windows instead of reusing one
  - Gang scroll images - move left and right images scrolling together
  - Gang zoom images - zoom left and right images scrolling together

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- Display gray values (C-G) - show gray values of cursor trial object
- Show report popup - display the report popup window again if needed

Landmark menu - define landmarks for spatial warping.
- Add landmark (C-A) - add trial objects (in images) as landmark
- Delete landmark (C-D) - delete the last landmark defined
- Show landmarks similarity - compute LSQ error measure of 2 sets of landmarks
- Set 3 pre-defined landmarks for demo images (C-Y)
- Set 6 pre-defined landmarks for demo images (C-Z)

Transform menu - perform various image processing transforms.
- Affine Warp - warp selected image using 3 pairs of landmarks
- Pseudo 3D transform - do pseudo 3D scaling based on image intensity

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- Sharpen Gradient - gradient + gray scale sharpen selected image
- Sharpen Laplacian - Laplacian + gray scale sharpen selected image
- Gradient - gradient of the selected image
- Laplacian - Laplacian of the selected image
- Average - average selected image
- Median - median of selected image
- Max 3x3 - max of 3x3 neighborhood of selected image
- Min 3x3 - min of 3x3 neighborhood of selected image

- Complement - complement selected image
- Threshold - threshold the selected image by gray values in [T1:T2]
- Contrast Enhance - Contrast enhance selected image
- Histogram equalize - histogram equalize selected image

- Original color - Restore original data for selected image
- Pseudo color - compute pseudo color scaling for selected image
- Color to grayscale - compute NTSC RGB to grayscale transform for image

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- Flip Image Horizontally - flip image horizontally selected image
- Flip Image Vertically - flip image vertically selected image

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- Repeat last transform (C-T) - repeat last transform, if any
- Use threshold inside [T1:T2] filter - filter pixels inside (outside) range

(*continued*)

Table 1 (*Continued*)
Summary of Commands Available in Flicker Menu

```
Quantify menu - contains OD calibration, background and foreground measurements.
  Measure circle ▶- measure intensity/density within circle
     Capture background (C-B) - background measurement at current position
     Capture measurement to spot list(C-M) - measure circle at current position
     ------
     Clear measurement - clear measurement data
     Edit selected spot(s) 'id' fields from spot list(s) (C-I)
     Edit selected spot(s) from spot list(s) (C-E)
     Delete selected spot from spot list (C-K)
     ------
     List spots in the spot list for selected image - measured spots report
     List spots in the spot list (tab-delim) - measured spots report for export
     List 'id'-paired annotated mean norm. spots in both spot lists (tab-delim)
     List 'id'-paired annotated spots in both spot lists (tab-delim)
     ------
     Lookup Protein IDs and Names in spot list from active map server
     ------
     Clear spot list (ask first) for selected image
     ------
  Print data-window ▶ - print the data window at the current image location
    Set print-window radix (C-V) - print gray-scale window popup report window
    Set print-window size ▶ - set print window size (5x5 to 40x40) pixels
    Set print-window radix ▶ - set data format (decimal, octal, hex, OD) radix
  ------
  Calibrate ▶ - calibrate optical density (OD) or other step wedge
    Optical density by step wedge - calibrate optical density from ND step wedge.
     Use demo leukemia gels ND wedge calibration preloads - to preset OD values
      ------
    Optical density by spot list wedge - calibrate OD from list of spots
  ------
  Region of Interest (ROI) ▶ - region of interest operations
    Set ROI ULHC (C-U) - define upper left hand corner of ROI
    Set ROI LRHC (C-L) - define lower right hand corner of ROI
    Clear (ROI) (C-W) - delete ROI
    Show (ROI) grayscale histogram (C-H) - for the current ROI
    Capture measurement by ROI (C-R) - measure integrated density less background
  ------
   Use sum density else mean density - region measurement method to use
   List-of-spots else trial-spot measurement-mode (C-J)

Help menu - popup Web browser documentation on Flicker from the Web server.
  Flicker Home - pop up Flicker home page open2dprot.sourceforge.net/Flicker
  Reference Manual - pop up the reference manual for Flicker application
  How-to use controls ▶ - pop up the references at particular manual sections
  Vignettes ▶ - pop up short vignettes showing how-to-do tutorials
  Version on the web site - show current version available on the Web site
  About Flicker - show details on Flicker application
  Old flicker applet documentation ▶ old flicker-applet Web home page
  2D gel web resources ▶ - useful 2D gel Web sites
```

Most often, the original images may be compared directly. However, occasionally, the comparison may be made visually easier by first applying enhancement transforms such as spatial warping, brightness, contrast, or other image transforms. Adjusting image brightness and contrast so the two gels have similar ranges will make the image

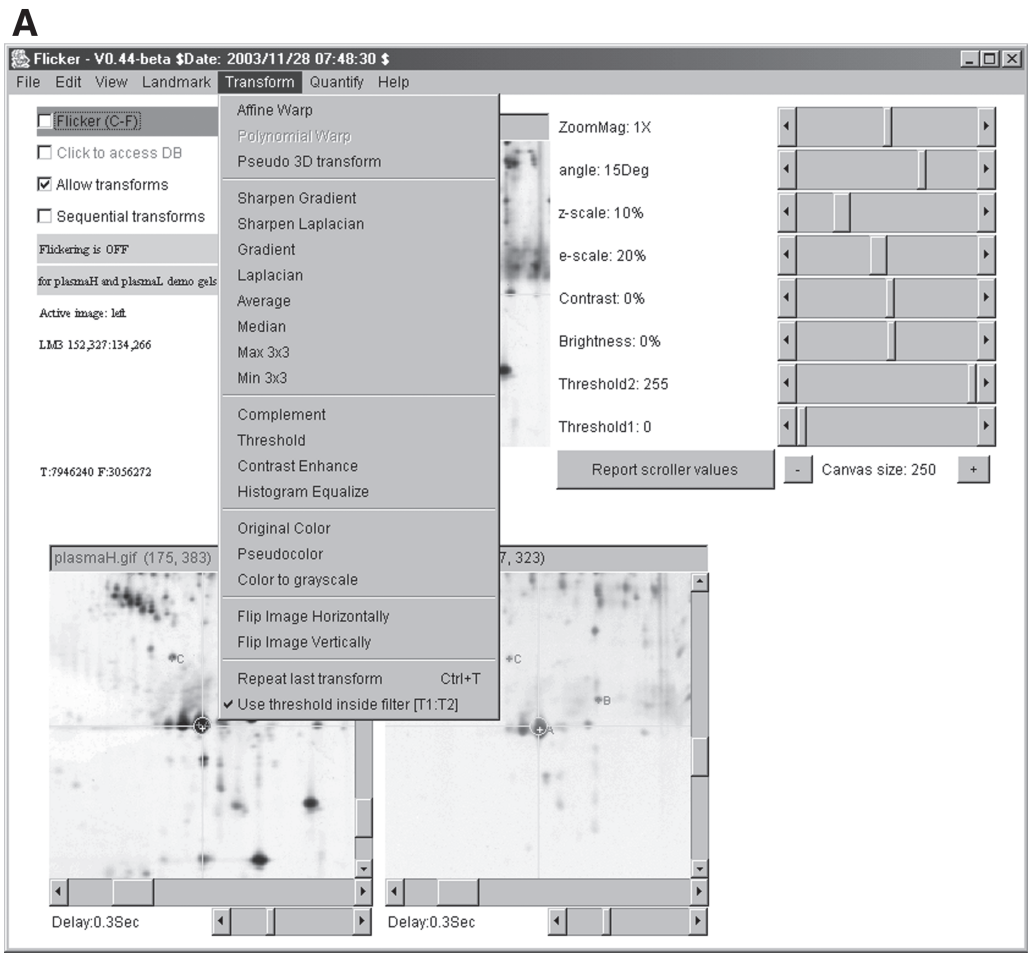

Fig. 2. Screen view of the landmarks used for the affine transform of the human plasma gel images. **(A)** The transform warps the geometry of a local region defined by the three landmarks so it more closely resembles the geometry of the corresponding local region in the other gel. Scrollable image windows with three "active" landmarks defined in both gel images that were selected interactively in preparation for doing the affine image transform. Corresponding landmark spots are selected so they are defined unambiguously in both gel images. For demonstration purposes, the command **Landmarks | Set 3 pre-defined landmarks for demo gels** will set up the three landmarks shown in this figure. **(B)** After defining the three landmarks, use the **Transform | Affine warp** command (*Fig. 2. continued on next page*).

fusion easier for the user when flickering. For gels with a lot of geometric distortion, it is useful to adjust the geometry of one gel so that the geometry of the local region being compared approximates that of the other gel. By local geometry, we mean the relative positions, distances, and angles of a set of spots in corresponding regions.

One technique to correct geometry differences is called "spatial warping." When performing spatial warping, corresponding regions of interest are (1) first marked by the user (we call this "landmarking") with several corresponding points in each gel

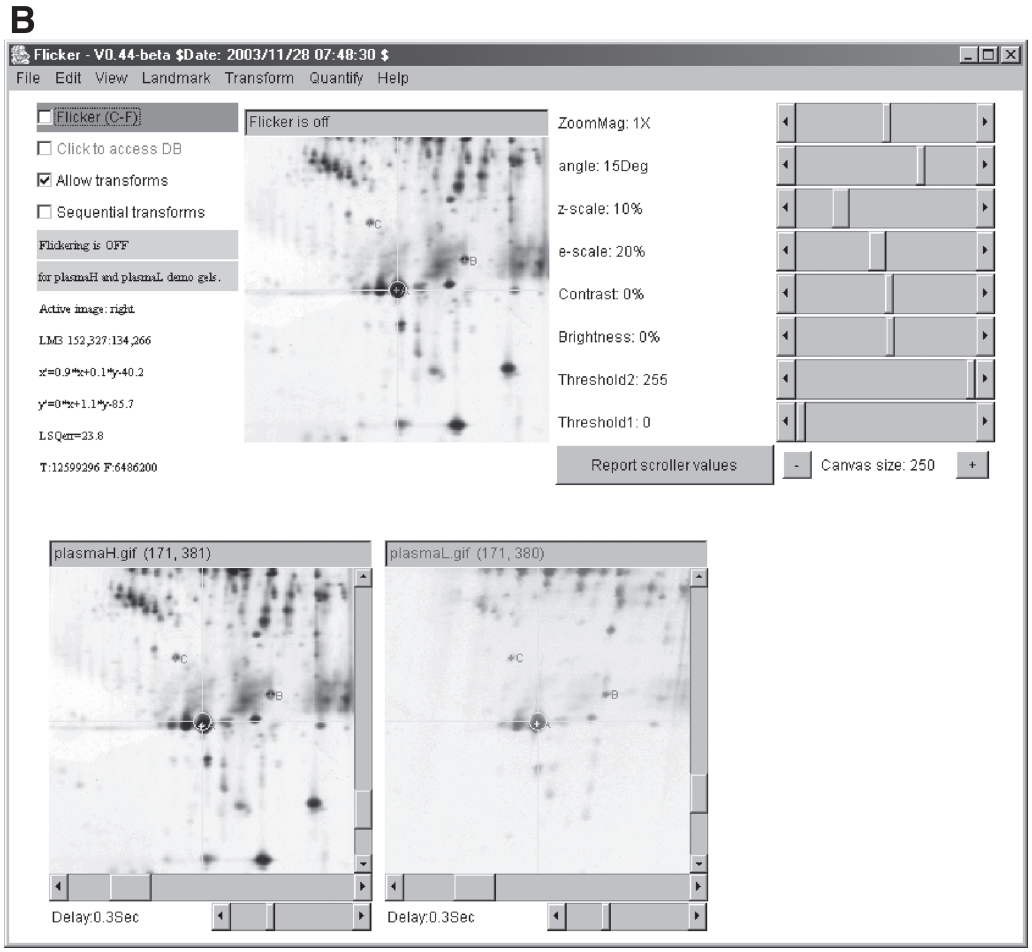

Fig. 2. (*continued*)

image (three for affine warping and six for poly warping), and (2) then one of the two gel images is warped to the geometry of the other gel (*see* **Equations 1** and **2**). A landmark is a corresponding spot that is present in both gels. Landmarks are defined by clicking on the spot to mark and selecting the **Landmark | Add Landmark (C-A)** command. The warping is performed by first selecting the image to warp by clicking on it, and then selecting the **Transform | Affine Warp** command. Landmarking and warping are described in more detail below.

Spatial warping doesn't change the underlying grayscale values of the synthesized warped image to the extent that would cause local structural objects to appear and disappear, possibly creating spot artifacts. Instead, it samples pixels from the original image to be transformed, and places them in the output image according to the geometry of the other input image. After warping is finished, gels may then be compared visually by flickering.

1.4. Image Flickering

The basic concept of using flickering as a dynamic visualization technique is simple. If two images may be perfectly aligned, then one could simply align them by laying one over the other and shifting one image until they line up. However, many images such as 2D PAGE gels have rubber-sheet distortion (i.e., local translation, rotation, and magnification). This means there is more distortion in some parts of the image than in others. Although it is often impossible to align the two whole images at one time, they may be locally aligned piece-by-piece by matching the morphology of local regions.

If it appears that a spot and the surrounding region do match, then one has more confidence that the objects are the same. This putative visual identification is our definition of matching when doing a comparison. Full identification of protein spots requires further work, such as cutting spots out of the gels and subjecting them to sequence analysis, amino-acid composition analysis, mass spectrometry, testing them with monoclonal antibodies, or other methods.

1.5. Image Enhancement

It is well known that 2-D gels often suffer from local geometric distortions, making perfect overlay impossible. Therefore, making the images locally morphologically similar while preserving their grayscale data may make them easier to compare. Even when the image subregions are well aligned, it is still sometimes difficult to compare images that are quite different. Enhancing the images using various image transforms before flickering may also help. Some of these transforms involve spatial warping, which maps a local region of one image onto the geometry of the local region of another image while preserving its grayscale values. Another useful operation is contrast enhancement, which helps when comparing light or dark regions by adjusting the dynamic range of image data to the dynamic range of the computer display. Other transforms include image sharpening and contrast enhancement. Image sharpening is performed using edge-enhancement techniques, such as adding a percentage of the gradient or Laplacian edge detection functions to the original grayscale image. The gradient and Laplacian have higher values at the edges of objects. In all cases, the transformed image replaces the image previously displayed. Other functionality is available in Flicker and is described in the Methods and Notes sections of this paper (*see* **Notes 3** and **12**), **Table 1**, and on the Web server.

1.6. Image-Processing Transforms

There are a number of different image transforms that may be invoked from the Transform menu. You may display the transformed image, use it as input to another transform, or save it as a .gif file on your local computer. When you save the state, you may also save the transformed images.

1.7. Affine Spatial Warping Transform

The spatial warping transforms require defining several corresponding landmarks in both gels. As we have mentioned, one gel image can be morphologically transformed to the geometry of the other using the affine or other spatial warping transformations. These transforms map the selected image to the geometry of the other image. It does not interpolate the grayscale values of pixels—just their position in the transformed

image. As described in ***(1–2,4–5)***, this might be useful for comparing gels that have some minor distortion, comparing local regions, gels of different sizes, or gels run under slightly different conditions. Flicker uses the affine transform as an inverse mapping, as described in ***(28)***. Let $(u_{xy},v_{xy}) = f(x,y)$, where (x,y) are in the output image, and corresponding (u,v) are in the input image. Then, in a raster sweep through the output image, pixels are copied from the input image to the output image. The affine transformation is given in equations 1 and 2:

$$u_{xy} = ax + by + c \quad (1)$$

$$v_{xy} = dx + ey + f \quad (2)$$

When the affine transform is invoked, Flicker solves the system of six linear equations for coefficients a, b, c, d, e, and f using three corresponding landmarks in each gel.

1.8. Pseudo 3D Transform

As described in **refs. *1,2,4,5***, the Pseudo 3D transform is a forward mapping that generates a pseudo-3D relief image to enhance overlapping spots with smaller spots, seen as side peaks. The gel size is width by height pixels. The gray value determines the amount of y shift scaled by a percentage z_{scale} (in the range of 0 to 50%). Pseudo perspective is created by rotating the image to the right (left) by angle theta (in the range of –45 to +45 degrees). The transform is given in equations 3–5 for image of size width × height, shift in the horizontal dimension computed as d_x.

$$d_x = \text{width} \sin(\text{theta}) \quad (3)$$

$$x' = (d_x (\text{height} - y)/\text{height}) + x \quad (4)$$

$$y' = y - z_{scale} \times g(x,y) \quad (5)$$

where g(x,y) is in the original input image and (x',y') is the corresponding position in the output mapped image. Pixels outside of the image are clipped to white. The Pseudo 3D transform is applied to both images so that one can flicker the transformed image.

1.9. Edge Sharpening

Edge sharpening may be useful for sharpening the edges of fuzzy spots. The sharpened image function g' (x,y) is computed by adding a percentage of a two-dimensional edge function of the image to original image data g(x,y), as shown in equation 6. The edge function increases at edges of objects in the original image and is computed on a pixel by pixel basis. Typical "edge" functions include the eight-neighbor gradient and Laplacian functions that are described in **refs. *1,2,4,5*** in more detail. The e_{scale} value (in the range of 0 to 50%) is used to scale the amount of edge detection value added.

$$g'(x,y) = (e_{scale} \times \text{edge}(x,y) + (100 - e_{scale}) \times g(x,y))/100 \quad (6)$$

1.10. Simple Quantitative Spot Measurement

A rudimentary spot measurement facility is available in which variably sized circles can be drawn around isolated spots and which allows for background correction. Lists of spots and paired spots may be created with annotation (*see* **Note 2**).

2. Materials

The following lists all items necessary for carrying out the technique. Because it is a computer technique, the materials consists of computer hardware, software, and an Internet connection. We assume the user has some familiarity with computers and the World Wide Web.

1. A Windows PC, MacIntosh with MacOS-X, a Linux computer, or a Sun Solaris computer having a display of at least 1024 × 768 resolution. At least 30 Mb of memory is required, and more is desirable for comparing large images or performing many transforms. If the computer does not have enough memory, it will be unable to load the images, the transforms may crash the program, or other problems may occur. An Internet connection is required to download the program from the http://open2dprot.sourceforge.net/Flicker Web site (*see* **Note 1**). New versions of the program will become available on this Web site and can be downloaded using the various Update commands described in the Notes section. If you have installer software that someone else downloaded and gave to you, then you do not need the Internet connection to install the program. If you will be using the active gel image maps associated with federated 2-D-gel databases, then you will need the Internet connection for accessing those databases. You do not need the Internet for local image comparisons. We currently distribute a version of Flicker that uses up to 128 Mb. If you want to run it with less or more memory, use the **Edit | Resize memory limits** command to set it to a value in the range of 30 Mb to 1784 Mb.
2. When you install Flicker, it creates several subdirectories (*see* **Notes 10** and **11**): *Images/* , containing the demonstration images; *DB/*, containing startup database files; and *FlkStartups/*, containing any startup files you create when you do a **File | SaveAs state file**. The *DB/* files are: *FlkDemoDB.txt*, which describes the demo images; *FlkMapDB.txt*, which describes the gel images and their corresponding active image map URLs, and *FlkRecentDB.txt* which lists recently accessed images. An empty database file *FlkRecentDB.txt* contains the file names and active gel map URLs, if any, of recently accessed images.
3. The Internet is a good source of 2-D-gel images. You can find them by searching WORLD-2DPAGE and 2D Hunt on the http://www.expasy.org/ server or a Google search to find other Web 2-D protein gel image databases. Links to these databases are available in the **Help | 2D gel Web resources** submenu.

3. Methods

We now describe the operation of Flicker from the user's point of view. You first install Flicker. Then run it with either the demonstration images, your own images, or images from the Internet. Then you simply flicker the gel images. If necessary, to improve the image comparability, use image enhancement transforms, before flickering the two images.

3.1. Installing Flicker From the Web Server

Click on the Download link on the http://open2dprot.sourceforge.net/Flicker Web site. This brings up the Java installer for your computer (we use the commercial InstallAnywhere installer by ZeroG.com). You may either click on the "Download Flicker for <computer type>" button or click on one of the links in the list of available installers. The latter is useful if you want to save the downloaded installer for later installation or for installing it on another computer. You have the option of downloading the "Java Virtual Machine (JVM)," which we recommend. This will not interfere

with any other JVMs you have already installed or may install in the future. Once the installer starts, you may select an installation language (English is the default) and press "OK." Then press the "Next" button after the Introduction window pops up. It then asks you where to install it, suggesting a reasonable default that you may override; then press the "Next" button. For Windows and some of the other systems, it will ask you where you want to put the startup icon; then press the "Next" button. After it finishes the installation, it will show the "Installation Complete" window. Finally, press the "Done" button to finish the installation. For example, in MS Windows systems, a "Flicker startup" icon will appear on your desktop.

To start Flicker, click on the startup icon. For Unix systems, including MacOS-X, you can start Flicker from the command line by specifying the path to *Flicker.bin*. Normally it comes up with the two demonstration human plasma 2-D-gel images—*plasmaH.gif*, an immobilized pH gradient (IPG) gel from SWISS-2DPAGE, on the left, and *plasmaL.gif*, a carrier ampholyte gel from Dr. Carl Merril/NIMH, on the right.

If you have your own gels (JPEG, GIF, or TIFF formats), you can try loading them. You may want to limit resolution by first decreasing their size using an image editing program like Photoshop or the shareware program ThumbsPlus (www.cerious.org). Large, very high-resolution images that are 20Mb to 40Mb will not work well. We suggest reducing the size to about 1K × 1K for good interactivity if you have any problems with running out of memory or very sluggish response. These image-editing programs can also be used for converting other formats to JPEG, GIF, or TIFF formats that Flicker can read.

3.2. Graphical User Interface for Flickering

Figure 1 shows the initial screen of the Flicker program. Pull-down menus at the top invoke file operations, edit preferences, view overlay options, landmarking, image transforms, and help commands. Scroll bars on the side determine various parameters used in the transforms. The two images to be compared are loaded into the lower scrollable windows. A flicker window appears in the upper middle of the screen. Checkboxes on the left activate flickering and control display options. A group of status lines below the checkboxes indicate the state of operations. **Table 1** shows the summary of the commands in the pull-down menus.

Only part of an image is visible in the scrollable windows. These subregions are determined by setting horizontal and vertical scroll bars. Another, preferred, method of navigating the scrollable images is to click on the point of interest while the Control key is pressed. This will re-center the scrollable image around that point. Note that if you are near the edge of the image when you do this, it will not scroll the image. This lets the user view any sub-region of the image at high resolution. These images may be navigated using either the scroll bars or by moving the mouse with the button pressed in the scrollable image window. Then, each image in the flicker window is centered at the point last indicated in the corresponding scrollable image window.

A flicker window is activated in the upper middle of the screen when the "Flicker" checkbox is selected. Images from the left and right scrollable images are alternatively displayed in the flicker window. The flicker delay for each image is determined by the adjusting the scroll bar below the corresponding scrollable image window. Various

graphic overlays may be turned on and off using the various view overlays selected in the **View | ☑ View …** checkbox menu commands.

Clicking on either the left or right image selects it as the image to use in the next transform. However, clicking on the flicker image window indicates the next transform you might use should be applied to both left and right images. You can change this default by just clicking on any of the images.

You can increase or decrease the size of the three image windows by using the **Edit | Canvas size | Increase size (C-keypad "+")** and **Edit | Canvas size | decrease size (C-keypad "–")** commands. This will resize the main window accordingly.

3.3. Loading Images

As mentioned in the introduction, gel images may be loaded into the left or right selected image from: (a) the local computer using the **File | Open image file** command; (b) any Internet site using the **File | Open image URL** command. You may load pairs of demonstration images that come with Flicker, installing them in the *Images/*directory. Use the **File | Open demo images | …** command to load them into the left and right images. The demos include a few samples that may be useful for initially learning the system. They include: two human plasma gels—an IPG SWISS-2DPAGE gel vs a carrier ampholyte gel (Merril/NIMH)—and some human leukemias (acute myeloid leukemia [AML], acute lymphoblastic leukemia [ALL], chronic lymphocytic leukemia [CLL], hairy cell leukemia [HCL]) from Lester et al. ***(9)***.

You may specify active gel images from the Web using the **File | Open active map image | …** to let you load one of the Swiss-2DPAGE gel images into the left or right selected image. This list of active images is defined by the tab-delimited *FlkMapDB.txt* file read by Flicker when it is started. "Power users" could edit this file (use Excel and save as tab-delimited) to add active map entries, pointing to other federated 2D-gel Web databases. The *FlkMapDB.txt* file is provided with your download installation in the *DB/* directory.

Gel images are loaded into the lower left or right images. First click on the left or right image you want to replace. Then, select the active gel image you want, using the **File | Open active map image | …** pull-down menu (e.g., select **… | SWISS-2DPAGE Human | Human Plasma**). Next, click on the other image and then open the other gel image you want to compare it with, using either the **File | Open image file** or the **File | Open image URL** command.

You may put directories of your own images to be compared in the Images subdirectory, and they will appear in **File | Open user images | ...** (*see* **Note 11**).

3.4. Flickering

When flickering two images with the computer, one aligns putative corresponding subregions of the two rapidly alternating images. The flicker window overlays the same space on the screen with the two images and is aligned by interactively moving one image relative to the other, using the cursor in either or both of the lower images. Using the mouse, the user initially selects what they suspect is the same prominent spot or object in similar morphologic regions in the two gel images. The images are then centered in the flicker window at these spots. When these two local regions come into

alignment, they appear to pulse and the images fuse together. At this point, differences are more apparent, and it is fairly easy to see which spots or objects correspond, which are different, and how they differ. We have found that the user should be positioned fairly close to the flicker window on the screen to optimize this image-fusion effect (i.e., it does not work as well standing back more than a few feet from the screen).

3.4.1. Selecting the Proper Time Delays When Flickering

The proper flicker delays, or the time each image is displayed on the screen, is critical for the optimal visual integration of image differences. We have also found that optimal flicker rates are dependent on a wide variety of factors, including amount of distortion, similarity of corresponding subregions, complexity and contrast of each image, individual viewer differences, phosphor decay-time of the display, ambient light, distance from the display, and so on. We have found the process of flickering images is easier for some people than for others.

When comparing a light spot in one gel with the putative paired darker spot in the other gel, one may want to linger longer on the lighter spot to make a more positive identification. Because of this, we give the user the ability to set the display times independently for the two images (typically in the range of 0.01 s to 1.0 s, with a default of 0.30 s), using separate Delay scroll bars located under each image. If the regions are complex and have a lot of variation, longer display times may be useful for both images. Differential flicker delays, with a longer delay for the light gel, are useful for comparing light and dark sample gels. This lets you stare at the lighter spots to have more verification that they are actually there.

3.5. Image Processing Methods

As mentioned before, there are a number of different image transforms that may be invoked from the menus. These are useful for changing the geometry, sharpness, or contrast, making it easier to compare potentially corresponding regions. As we go through the transforms, we will indicate how they may be used. Some affect one image, while some affect both. Flickering is deactivated during image transforms to use most computational power for doing the transforms.

The Transform menu has a number of commands, which include warping, grayscale transforms, and contrast functions. The two warp method selections—"Affine Warp" and "Poly Warp"—are performed on only one image (the last one selected by clicking on an image). The "Pseudo 3D" transform makes a 3-D image with the "peaks" created proportional to gray level. Unlike the warp transforms, the grayscale transforms are performed on both images. These include: "SharpenGradient," "SharpenLaplacian," "Gradient," "Laplacian," "Average," "Median," "Max 3×3," and "Min 3×3." The contrast functions are "Complement" and "ContrastEnhance." You can transform color images to grayscale using the "Color to grayscale" command, and generate a false color image from a grayscale using the "Pseudo color" command. You can flip the image using "Flip image horizontally" or "Flip image vertically" commands.

3.5.1. Landmarks: Trial and Active

The affine transform requires that three active landmarks be defined before it can be invoked. A trial landmark is defined by clicking on an object's center anywhere in a scrollable image window. This landmark would generally be placed on a spot. Clicking

on a spot with or without the Control key pressed still defines it as a trial landmark. After defining the trial landmark in both the left and right windows, selecting the **Landmark | Add Landmark (C-A)** command defines them as the next active landmark pair and identifies them with a red letter label (+A, +B, +C, …) in the two scrollable image windows. The **Landmark | Delete Landmark (C-D)** command is used for deleting the last landmark you defined.

3.5.2. The Affine Transform for Spatial Warping

The two warping transforms, affine (*see* **equations 1** and **2**) and polynomial, require three and six landmarks, respectively. Attempting to run the transform with insufficient landmarks will cause Flicker to notify you that additional landmarks are required. The image to be transformed is the one last selected. You must select either the left or right image. **Figure 2A** shows the landmarks the user defined in the two gels before the affine transform. **Figure 2B** shows the transformed image. Then, re-center the transformed image before you flicker. After the transform, the landmarks can be lined up perfectly, and adjacent spots will line up better.

3.5.3. Pseudo 3D Transform

As described in **refs. *1,2,4,5*** and as shown in **equations 3–5**, the Pseudo 3D transform generates a pseudo–3-D relief image to enhance overlapping spots, with smaller spots seen as side peaks. The gray value determines the amount of *y* shift scaled by a percentage (set by scroll bar z_{scale}) in the range of 0 to 50%. Pseudo perspective is created by shifting the image to the right or left by setting by scroll bar "angle" degrees (in the range of –45 to +45 degrees). Negative angles shift it to the right and positive angles to the left. The image to be transformed is the one last selected. If neither was selected (i.e., you clicked on the flicker window), then both images are transformed.

3.5.4. Edge Sharpening

Edge sharpening may be useful for improving the visibility of the edges of fuzzy spots. You can select either a Gradient or Laplacian edge-sharpening function using the "SharpenGradient" or "SharpenLaplacian" operation in the Transform menu where the image to be transformed is the one last selected. The Laplacian filter generates a "softer" edge than the Gradient. You can set the scroll bar e_{scale} value (in the range of 0 to 50%) to scale the amount of edge detection value added. The image to be transformed is the one last selected. If neither was selected (i.e., you clicked on the flicker window), then both images are transformed.

*3.5.5. Putative Identification of a Spot in One Gel by Comparison With Federated Database Gel Map (*see ***Fig. 3****)*

Au: Fig. citation ok?

Open an active gel image in the lower left or right window. First click on the window you want to load the new image. Then, select the active gel image to you want, using the entry **File | Open active map image | …** pull-down menu (e.g., select **… | SWISS-2DPAGE Human | Plasma**). Next, click on the other image and then use the other gel image you want to compare it with, using one of the other **File | Open …** commands.

At this point, flicker the two images so that you can make a putative guess as to which spot you are interested in—which spot in the active map gel your gel corre-

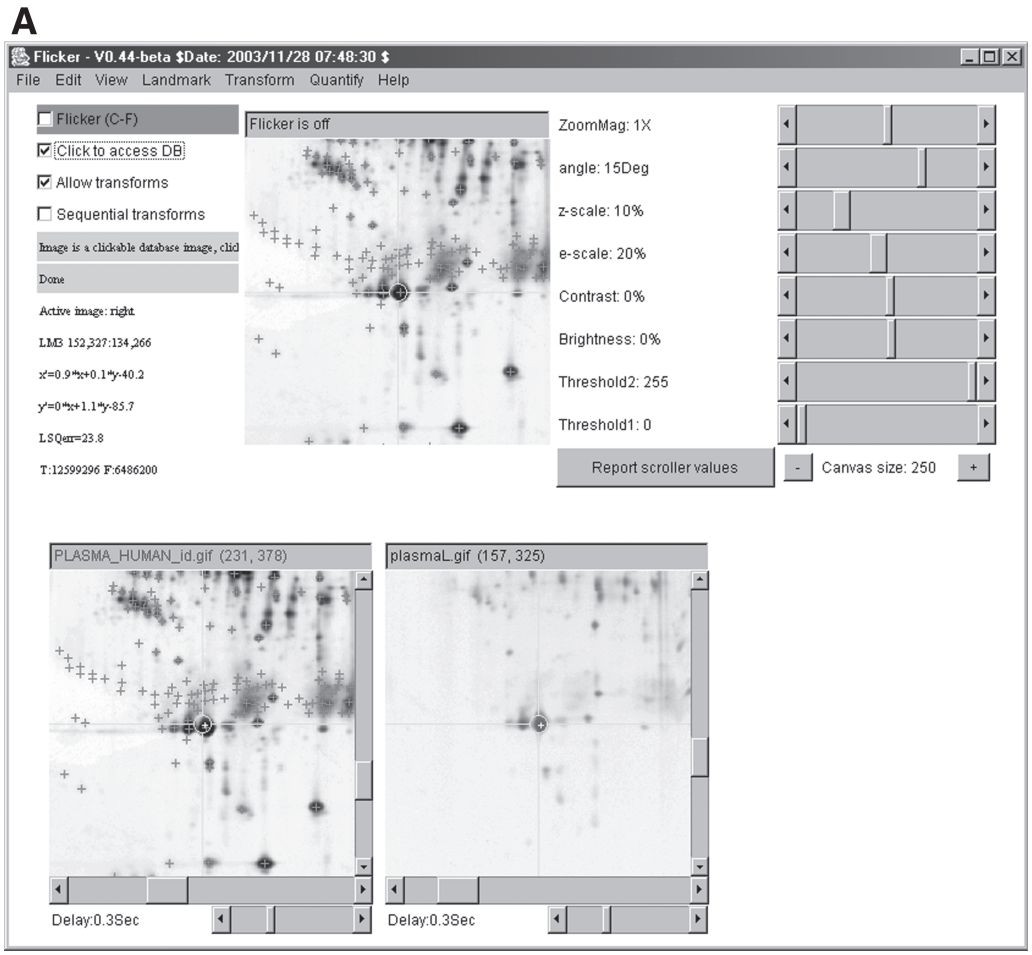

Fig. 3. Screen views of clickable active gel compared with another gel. **(A)** We have loaded the active SWISS-2DPAGE human plasma gel (PLASMA_HUMAN_id) in the left image and the plasmaL gel in the right image. Spots that appear in SWISS-2DPAGE are indicated with red "+" symbols. We then aligned the images using flickering. We then selected the "Click to access DB" checkbox. Finally, we clicked on the indicated spot in the left gel to determine the putative identification of the corresponding spot in the right gel. **(B)** The SWISS-2DPAGE window then popped up as a result of clicking on that spot in the left image, and indicates the putative protein identification of the visually corresponding spot in the right gel. The plasmaH image is the same gel as PLASMA_HUMAN_id but without the graphic overlays. You can load these same gels using the **File | Open demo images | Human Plasma gels | (SWISS-2DPAGE vs Merril)—clickable** command, which should be used when you are connected to the Internet.

sponds to. Then, shut off flickering by turning off the "Flicker" checkbox. Then, turn on the Click to access DB checkbox. Then, click on the spot in the active map image, which will pop up a Web-browser window indicating the SWISS-2DPAGE Web page for that spot, if it is in their database.

B

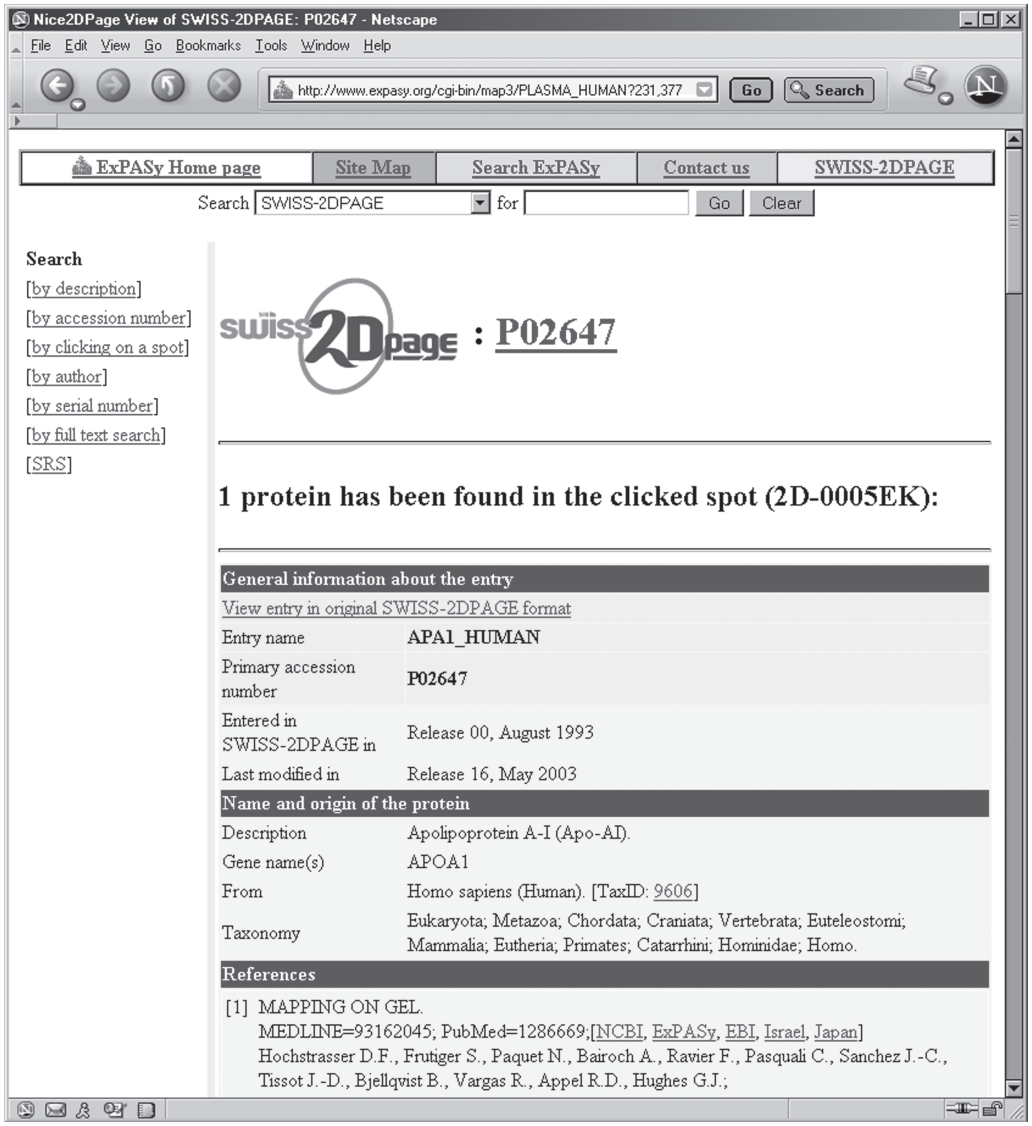

Fig. 3. (*Continued*)

4. Notes

4.1. Status of the Flicker Application

As of the time of the submission of this chapter, most of the functionality available in the former Java applet ***(1,2,4,5)*** is fully functional in the stand-alone application. The current state of Flicker is documented on the Web server. A future release of Flicker will contain a spot quantification function, with the ability to calibrate the image (either from the calibration information in the image itself, if available, or from a scanned neutral-density step wedge scanned with the image), estimate background density, and

estimate spot intensity with a background subtraction. Documentation is available on the Web site. This documentation may also be invoked from the Help submenus.

The original Flicker program was converted from a Java applet to a Java application by Peter Lemkin and Greg Thornwall, with help from Jai Evans. Code was added from the open source MicroArray Explorer (http://maexplorer.sourceforge.net/) program. The new Flicker program uses the Mozilla 1.1 open source license and is available on the open source Web server http://open2dprot.sourceforge.net/Flicker/.

You can update your program and data files using the various Update options in the Files menu. The **File | Update | Update Flicker Program** command downloads and installs the latest *Flicker.jar* file. The **File | Update | Update active Web maps DB** command downloads and installs the latest active Web maps database *DB/FlkMapDB.txt* file. The **File | Update | Update Demo images DB** command downloads the latest demo images into the *Images/* directory.

4.2. Hints on Measuring Spots

There are some disadvantages in comparing gels visually. It is useful for doing a rough comparison, and there is currently no simple way available to do adequate quantitative comparison (as can be done with existing 2-D-gel computer database systems) using automatic spot segmentation and global normalization methods. However, you can look at the gray value of the cursor in the left or right image if you enable the **View | ☑ Display gray values** menu option. We are working on an extension to the program to allow single-spot quantification with optical-density calibration. This will be announced on the Flicker Web site. These limitations should be kept in mind when using the technique.

In the meantime, we are providing a simple method that can be used for ballpark estimates of density if your gels and scanner are reasonably linear, so that grayscale approximates protein concentration. You can do this in either of two ways: by measuring the area under a circular mask (you can set the radius), or the area inside a rectangular region of interest (ROI). Note that unless the spot fits well inside of the mask or ROI, you will not get a very accurate measurement. Both methods can subtract an optional background value you can capture and so can give intensity corrected for background if defined.

Use the "measure circle" slider to set the measurement circle region size (1×1, 3×3, 5×5... or 51×51. Click on a background region near where you want to measure a spot's density within a circular mask. Then select the **Quantify | Measure by circle | Capture background (C-B)** command. Then click on the center of the spot you want to estimate and select the **Quantify | Measure by circle | Capture measurement (C-M)** command. This will compute and display background-corrected data that appear in the report window as:

```
Setting the circle size to: 9 × 9
plasmaL.gif (83, 278) Bkgrd value: 670 tot mask(9 × 9) gray-value
plasmaL.gif (156, 323) Tot(Meas-Bkgrd): 5452 gray-value
TotMeas: 6122 [143:162], TotBkgrd: 670 at (83, 278) [14:20] gray-value
CircleMask: 9 × 9
```

The **View | ☑ View measurement circle** displays the background and foreground measurement circles, if enabled, with "B" and "M" labels. Set the **View | ☑ Use sum**

density else mean density menu option to specify that it report either total region density or mean density.

You also can measure intensity inside a rectangular ROI region you set by using both the **Quantify | Region of Interest (ROI) | Set ROI ULHC (C-U)** and the **Quantify | Region of Interest (ROI) | Set ROI LRHC (C-L)** commands. Use the **View | ☑ View Region Of Interest (ROI)** to display the ROI as a rectangle from the ULHC to the LRHC (upper left-hand corner and lower right-hand corner). You can measure the integrated density of the **Quantify | Region of Interest (ROI) | Capture measurement by ROI (C-R)** command. If you set it, the (C-R) command will subtract the background computed by the area times the mean background using the **Quantify | Measure by circle | Capture background (C-B)** command. This will compute and display background corrected data that appear in the report window as:

```
Setting ULHC (188,234) of ROI: right image
Setting LRHC (211,256) of ROI: right image
plasmaL.gif (211, 256) Tot(Meas-Bkgrd): 5936.923 gray-value
TotMeas: 15420 [15:127], TotBkgrd: 9483.077 at (83, 278) [14:20] gray-value
ROI: [188:211, 234:256]
```

When grayscale calibration is added in a future version, then the measurements will be in terms of the calibration rather than grayscale.

4.3. Additional Hints on Image Transforms

The intent of applying image transforms is to make it easier to compare regions that have similar local morphologies but some different objects within these regions. Image warping prior to flickering is intended to spatially warp and rescale one image to the geometric "shape" of the other image so that we can compare them at the same scale. This should help make flickering of some local regions on quite different gels somewhat easier. Of the two warping transforms, affine and polynomial, the latter method handles non-linearities better. For those cases where the gels are similar, the user may be able to get away with using the simpler (affine) transform. For demonstration purposes, if you are using the demo *plasmaH* and *plasmaL* gels, the **Landmark | Set 3 pre-defined landmarks for demo gels (C-Y)** and **Landmark | Set 6 pre-defined landmarks for demo gels (C-Z)** define three and six corresponding landmarks for these gels that may be used with the affine and polynomial warping transforms, respectively.

In cases where there is a major difference in the darkness or lightness of gels, or where one gel has a dark spot and the other a very faint corresponding spot, it may be difficult to visualize the light spot. By differentially setting the flicker display-time delays, the user can concentrate on the light spot using the brief flash of the dark spot to indicate where they should look for the light spot. We have found differential flicker to be very helpful for deciding difficult cases. Adjusting one image so that its brightness and contrast are approximately that of the other image also helps when flickering. You change the image brightness and contrast using the Shift/Drag mouse control described in **Note 4.5.**

Other transforms including image sharpening may be useful in cases where spots are very fuzzy, as might be the case when comparing Southern blots. When two corre-

sponding local regions of the two images are radically different so the local morphologies are not even slightly similar (e.g., when high-MW regions of gels are run differently, such as: IPG vs non-IPG, gradient vs nongradient sodium dodecyl sulfate [SDS]), then even using these transforms may not help that much.

4.4. Saving and Restoring the Flicker State

Flicker gives you the option of saving the current state of your session, including the images you are looking at and the parameter values of the sliders, and so on. To save the current state, use the **File | Save (or SaveAs) state file** command. This creates a file with a .flk file extension in the installation *FlkStartups/* folder (default *FlkStartup.flk).* If you have used the Flicker Web site Java installer (ZeroG.com) for installing Flicker, then it lets you click on a specific *.flk* you have previously saved to restart it where you left off. While running Flicker, you can also use **File | Open state file** command to change it to another state.

4.5. Mouse Control of Images

The following mouse and key-modified mouse operations control various actions.

Pressing the mouse in either the left or right image selects it. If flickering is active, then it will move the center of the flicker image for the selected image to that position. A little yellow "+" indicates the position you have selected. If the "Click to access DB" checkbox is enabled and the image has an associated active map database server

associated with it, then it will request the spot identify when you click on a spot from the map database.

Dragging the mouse is similar to pressing it. However, only pressing it will invoke a clickable database. It also displays the cursor coordinates in the image title.

Control/Press will position the selected image so that the point you have clicked on will be in the center of the crosshairs. If you are near the edge of the image, it will ignore this request.

Shift/Drag activates the brightness/contrast filter with minimum brightness and contrast in the lower left-hand corner.

4.6. Checkbox Control of Flickering and Database Access

There are four checkboxes in the upper left part of the window that control commonly used options.

The "Flicker" checkbox enables/disables flickering.

The "Click to access DB" checkbox enables/disables access to a Web database server that is associated with a clickable image, if it exists for the selected image. Turning on this option will disable flickering.

The "Allow transforms" checkbox enables/disables image transforms.

The "Sequential transforms" checkbox enables/disables using the last image transform output as input for the next image transform (image composition) if "Allow transforms" is enabled.

4.7. Keyboard Shortcut Controls

There are several short-cut key combinations that can be used to perform operations instead of selecting the command from the pull-down menus. The notation **C-<*key*>** means to hold the Control key (the Apple key on the Macintosh) and then press the following <*key*>.

- **C-A**—add landmark (you must have selected both left and right image trial objects) (see landmark menu)
- **C-B**—capture background intensity value for current image under circle (see quantify menu)
- **C-D**—delete landmark (the last landmark defined; see landmark menu)
- **C-F**—toggle flickering lower left and right images into the upper flicker window (see view menu)
- **C-G**—toggle displaying gray values in the left and right image titles as move the cursor (see view menu)
- **C-H**—show grayscale ROI histogram. Popup a histogram of the computation region of interest (ROI) (see quantify menu)
- **C-I**—Define or edit selected measured spot(s) annotation "id" field (see quantify menu)
- **C-J**—toggle the spot measurement mode between list-of-spots measurement mode and the single spot trial-spot measurement mode (see quantify menu)
- **C-K**—delete selected measured spot, click on spot to select it (see quantify menu)
- **C-L**—define lower right hand corner (LRHC) of ROI and assign that to computing window (see quantify menu)
- **C-M**—measure & show intensity under circle for current image, report background corrected value defined (see C-B shortcut and quantify menu)
- **C-R**—measure and show intensity under a the computing window defined by the ROI (see C-U and C-L) for current image. Report background-corrected value if circular mask background was defined (see C-B shortcut and quantify menu)
- **C-T**—repeat the last Transform used, if one was previously performed else no-op (see transform menu)
- **C-U**—define upper left hand corner (ULHC) of ROI and assign that to computing window (see quantify menu)
- **C-V**—show data-window of selected pixel in the popup report window (see quantify menu)
- **C-W**—clear the ROI and computing window (see quantify menu)
- **C-Y**—set 3 predefined landmarks for demo gels for Affine transform (see landmark menu)
- **C-Z**—set 6 predefined landmarks for demo gels for Polywarp transform (see landmark menu)
- **C-Keypad "+"**—increase the image canvas size for all three images (see edit menu)
- **C-Keypad "-"**—decrease the image canvas size for all three images (see edit menu)

4.8. Reporting the Status in the Pop-Up Status Window

Information is display in several places in Flicker:

(a)There are two status lines in the upper left part of the main window. The output into these status lines is also appended to the Report window (c).
(b) The selected image (clicking on the left or right image) changes its title to blue from black. If neither image is selected, then both titles are black.
(c)A report pop-up window is created when Flicker is started. It may be temporarily removed by closing it. You can get it back at any time by selecting **View | ☑ Show report popup**. All text output is appended to this window. The Clear button clears all text. The SaveAs button lets you save the text in the window into a local text file.

4.9. Sliders for Defining Parameters

The following sliders are in the upper right part of the window and are used for adjusting parameters in the various image transforms:

angle (degrees) used in the Pseudo 3D transform
brightness (%) set by Shift/Drag to change the image brightness
contrast (%) set by Shift/Drag to change the image contrast
eScale(%) used in the sharpening transforms
threshold1 (grayscale or od) is the minimum grayscale value to show pixels otherwise they are shown as whites
threshold2 (grayscale or od) is the maximum grayscale value to show pixels otherwise they are shown as white
zoomMag (X) to zoom both left and right images from 1/10X to 10X by a transform
zScale (%)used in the Pseudo 3D transform

4.10. Local Database Files

When Flicker is installed, several tab-delimited (spreadsheet derived) *.txt* files are available in the *DB/* directory (located where the *Flicker.jar* file is installed). These *DB/Flk*DB.txt* files are read on startup and are used to set up the **File | Open ... image | ...** menu trees:

DB/FlkMapDB.txt—contains instances of Web-based active image maps with fields: *MenuName, ClickableURL, ImageURL, BaseURL, DatabaseName*

DB/FlkDemoDB.txt —contains instances of pairs of images in the local Images/ directory and contains fields: *SubMenuName, SubMenuEntry, ClickableURL1, ImageURL1, ClickableURL2, ImageURL2, StartupData*

DB/FlkRecentDB.txt—contains instances of recently accessed non-demo images with fields: *DbMenuName, ClickableURL, ImageURL, DatabaseName, TimeStamp*

4.11. Files Required That Are Included in the Download

The following files are packaged in the distribution and installed when you install Flicker:

Flicker.jar is the Java Archive File for Flicker that is executed when you run Flicker.
jai_core.jar is the core Java runtime from SUN's Java Advanced Imaging (JAI) at sun.com.
jai_codec.jar is the JAI tiff file reader from SUN's Java Advanced Imaging JAI at sun.com.
DB/ is a directory containing the set of tab-delimited DB files *Flk*DB.txt* read at startup.
Images/ is a directory holding demo .gif, .tif, .jpeg, and .ppx sample files as well as a user's subdirectories of images.
FlkStartups/ is empty directory into which to put the startup *FlkStartup.flk* files.

4.12. Image Transform and Brightness-Contrast Display Model

There are several display models for combinations of using image transforms, zooming, and brightness/contrast filtering. Zooming is an image transform, and you can de-magnify as well as magnify. These transforms and filtering are applied to the left and right windows and also are shown in the flicker window. Two checkboxes in the upper left of the main window control transforms: "Allow transform" enables/disables transforms, and "Sequential transforms" allows using the previous transform as the input to the next transform—i.e., this lets you implement image composition.

This description applies independently to the left and right images. The original image is denoted *iImg*. If you allow transforms and are also composing image trans-

forms, you may optionally use the previous transformed output image (denoted *oImg*) as input to the next image transform. The output (either *iImg* or *oImg*) is then sent to the *output1*. Then *output1* may be optionally zoomed to *output2* by being sent to the zoom transform (if the magnification is different from 1.0X). Then *output2* may be optionally contrast adjusted by being sent to the brightness-contrast filter (if it is active, as specified by dragging the mouse in the selected window with the Shift key pressed). The *output2* of the brightness-contrast filter is denoted as *bcImg*. If you have not used the zoom or brightness-contrast filtering since loading an image, then *zImg* and *bcImg* are not generated and hence not used in the displayed image. This will speed up display refresh as you navigate the windows.

(a) If no transforms or brightness-contrast filtering is used on the selected image

(no transforms)

iImg → output1

(b) The image may be optionally transformed from the original image (*iImg*)

(transform)

iImg → oImg → output1

(c) Image transforms may be optionally composed from the original image or from the sequential composition of image transforms on the selected image

(sequential transforms)

iImg → Transform → oImg → output1

(d) The image may be optionally zoomed if the magnification is not 1.0X

(zoom)

output1 → zImg → output2

or

(no zoom)

output1 → output2

(e) The brightness-contrast filter may be optionally applied to the image

(B-C filter)

output2 → bcImg → display

or

(no B-C filter)

output2 → display

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