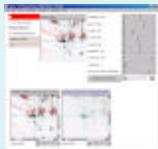


## Flicker Comparison of 2D Electrophoretic Gels

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<http://open2dprot.sourceforge.net/Flicker>

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## Overview

- Flicker is an open-source stand-alone Java computer program for visually comparing 2D gel electrophoresis images
- 2D polyacrylamide gel electrophoresis (2D-PAGE) gels are often difficult to compare because of rubber-sheet distortions
- Flicker allows you to compare your gel images against each other or against those found in Internet databases

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## Overview (continued)

- Many published Internet gels have subsets of spots identified which may make them useful to compare with your gels.
- You might draw putative conclusions to identification of some spots in your gels that visually appear to be the same spots as in reference gels

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## Main Features of Flicker

- Flicker allows comparison of two gel images at a time
- Menu system helps organize and access sets of local user gels and access Internet reference database gels
- Access SWISS-2DPAGE active map reference gels database. Extendible to other federated databases
- Image enhancement helps support visual comparison: zoom, brightness/contrast, spatial-warping, smoothing, sharpening

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## Main Features (continued)

- Create lists of spot measurements for estimating spot quantification and doing annotation
- Calibrate gray scale if OD, CPM, etc. standards are available
- Export measured spot lists and annotated paired spot lists (to Excel, etc.)
- Save/restore data-mining sessions

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## Availability of Flicker

- Written in Java as open source and is freely available at <http://open2dprot.sourceforge.net/Flicker>
- Runs on MS Windows, MacOS-X, Linux, Solaris
- Download and install using a standard installer
- Documentation as HTML or PDFs
- Tutorial vignettes on using Flicker

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Home page: <http://open2dprot.sourceforge.net/Flicker>

In Table of Contents:

- \* [Introduction](#)
- \* [Reference Manual](#)
- \* [Vignettes](#)
- \* [Download](#)
- \* [Other Web resources](#)

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### Flicker Program User Interface

Parameter sliders

Flicker, transform, database controls

Flicker window

Two scrollable images specified by user

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### Image Sources – Active Map Gel Images

Select Web reference sample

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### Image Sources – Local User Gel Images

Or, select directory of single images

Select directory of pairs of images

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### Image Sources – Demo Gel Images

Select demo gel images

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### The Image-Comparison Problem

- If two images *could* be perfectly aligned then one could simply align them by overlaying one over the other and shifting one image until all objects in the images line up
- Many images such as 2D PAGE gels have non-linear rubber-sheet distortion
- There may be more distortion in some parts of the images than in others
- However, some images may be locally alignable
- Warping local regions, (e.g., affine linear transform of translation, rotation, and magnification) can further improve local comparison

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## Concept of Flicker-Comparison

- Flickering is a dynamic visualization technique for overlaying two images
- Although it may be impossible to align two whole images at one time, they may be locally aligned piece-by-piece by matching the morphology of local regions
- Alternating two images in the same visual space will "fuse" the aligned regions in your minds-eye when they are optimally aligned

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## Problems with Flicker-Comparison

- Because flickering is a dynamic visualization technique that depends on hand-eye-brain integration, we find that some people are better at using this technique than others
- There is the occasional danger of false alignments when comparing charge-trains of spots if there is not enough local morphology context
- Because it may be difficult to compare a user's entire gel against an Internet database reference gel (e.g., Swiss-2DPAGE) which was run in a quite different way:
  - IPG vs Carrier-Ampholytes,
  - linear vs non-linear gradients,
  - ple isoelectric ranges,
  - MW molecular mass ranges,
  - etc.

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## Problems with Flicker-Comparison (continued)

- However, parts of the gels may be comparable
- Even when a comparison is made and a putative correspondence found between the user's and the reference gel, the spot of interest may not have been identified in the Internet database reference gel

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## Other Image-Alignment Methods

- DIGE (using Cy -type dyes) to run simultaneous samples. Has a max 5 samples and difficult to compare images between labs if samples not run simultaneously
- Warp two images completely that are different colors. There may be problems warping gels that are not very similar

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## Flicker Image Transforms for Better Visualization

Sometimes, it is difficult to visually compare gels of different magnification, contrast, and geometry – Flicker has methods to help

- Zoom to magnify or de-magnify a gel closer to the magnification of other gel
- Brightness-contrast adjustment to adjust one gel to range of the other gel
- Local geometric correction using spatial warping
- Image enhancement available for smoothing and sharpening images

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## Transform Menu and Parameter Adjustment

The screenshot shows a software interface with a 'Transform' menu open. The menu options include: 'Zoom', 'Brightness/Contrast', 'Local Geometric Correction', 'Image Enhancement', 'Smoothing', 'Sharpening', 'Canvas Size', and 'Circle Size'. A yellow callout box points to the 'Image Enhancement' option, stating 'Image enhancement transforms'. Another yellow callout box points to the 'Image Enhancement' parameter sliders, listing: 'brightness/contrast', 'zoom', 'canvas size', 'circle size', and 'etc.'.

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### Finding Putative Identifications by Accessing Reference 2D Web Databases

1. First find a putative match between a spot in a user's gel and one in an active map reference gel
2. Then user selects the spot in the reference gel to access that spot's identification in the Internet reference gel database
3. The reference Web site database can supply the identification of the selected spot and by inference the putative identification of the user's spot

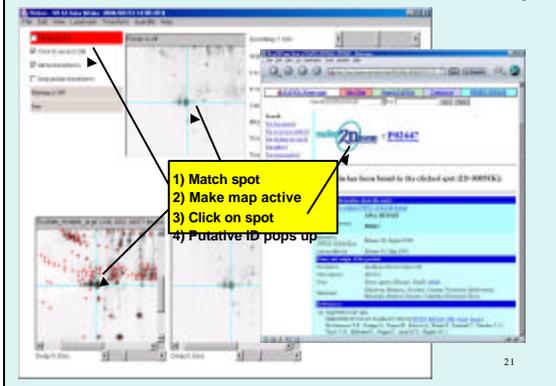
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### Finding Putative Identifications by Accessing Reference 2D Web Databases (continued)

4. An active map reference gel must be supported by a federated 2D gel map Internet database such as Swiss-2DPAGE or Flicker won't be able to access it
5. Additional lab work might be needed to confirm putative identifications of the spots extracted from a user's gel(s)

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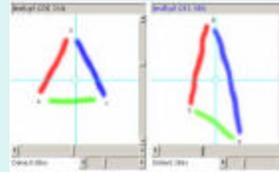
### Putative Identification - Click on Active Map



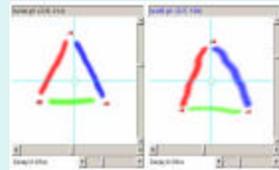
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### Warping a Gel to Other Gel's Geometry

Original



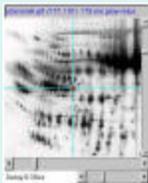
Warped



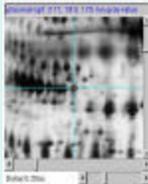
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### Zooming a Gel to Other Gel's Magnification

Original



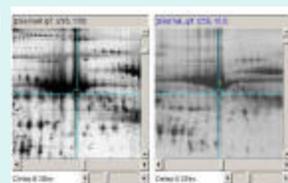
Zoomed



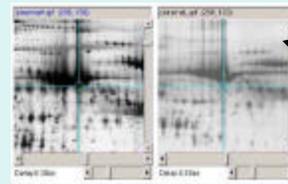
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### Adjusting Brightness/Contrast So Similar

Original



Adjusted



Similar brightness and contrast

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## Estimating Spot Quantification

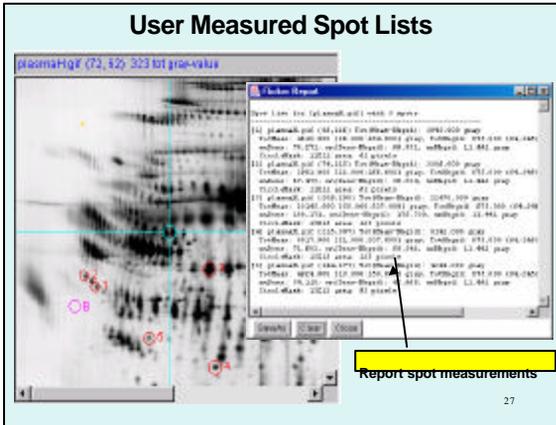
- Flicker provides a **limited** estimated-spot quantification capability to collect a list of manually-measured isolated-spots
- Integrated density (grayscale or calibrated OD) may be **estimated** for isolated spots using measurement circle masks (1 to 51 pixels in diameter)
- Background density,  $D_b$ , near the spot is measured first
- Then, an isolated spot's density,  $D_s$ , is measured and the density corrected for background  
 $D'_s$  is estimated as  $D_s - D_b$
- Lists of spots may be created with user-supplied annotation
- Spot-lists be reported and saved for further analysis

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## Spot Quantification Menu



## User Measured Spot Lists



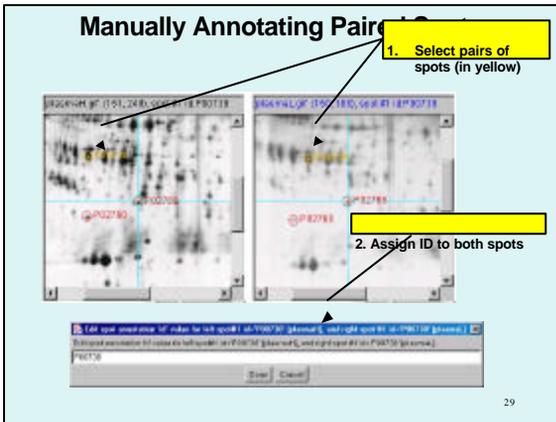
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## Spot List Editing Functionality

- Define, delete, annotate, edit spots in the spot list (per gel)
- View spots with various overlay options
- List spots in report-form or tab-delimited form suitable for export to Excel or other analysis programs
- List paired-spots reports with same IDs in tab-delimited form
- Save gel names, spot lists and other parameters in a state file for further use when exit Flicker and reload them when reload the state file

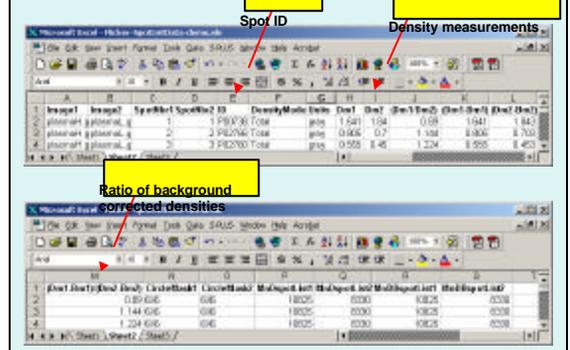
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## Manually Annotating Paired Spots



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## Generate Paired-Spots Reports For List of Spots with Same IDs



## Looking Up Paired-Spot ID Annotation at the Reference Database

Putatively identify a list of spots in your gel that are identified in an active reference gel by first identifying spots in the reference gel and then using them to identify corresponding spots in your gel sample.

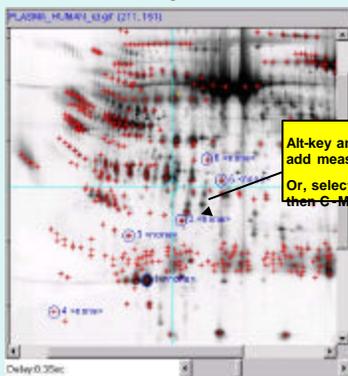
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## Procedure: Looking Up Paired-Spot ID Annotation

1. Open 2 gels to compare (pick one as an active reference gel).
2. Flicker align similar regions for each of the spot(s) of interest.
3. Add spots of interest to spot lists (a separate list for each gel).
4. Request Flicker visit the active reference gel Web server and try to look up the protein names and IDs (e.g., SWISS-PROT) for the spots you have defined in the active gel.
5. Repeatedly select a pair of corresponding spots in 2 gels, and pair them using a common annotation id from the reference gel.
6. List the spots in the paired spot list (this can be generated as tab-delimited data for export to Excel).

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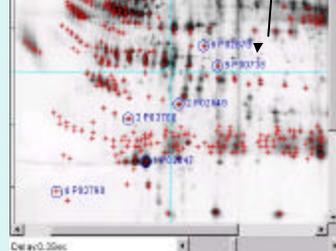
## First, Define Spot List in Reference Gel



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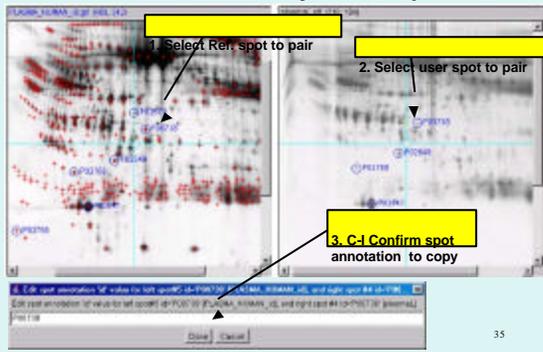
## Then, Look Up Reference Gel Spot Ids & Names at SWISS-2DPAGE

Look up all protein IDs in spot-list at SWISS-2DPAGE. See (Quantify | measure by circle | Lookup Protein Ids...) menu command



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## Finally, Assign Reference Gel Annotation to User Gel for Manually Paired Spots



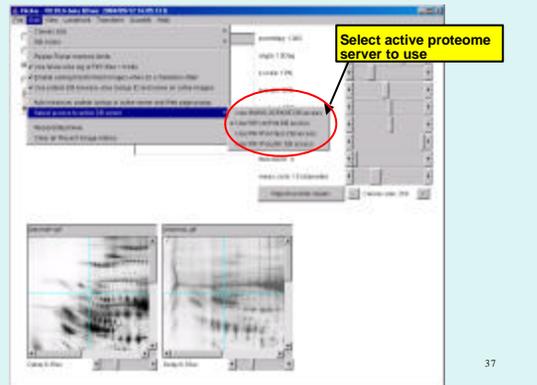
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## Lookup PIR Web Pages for Identified Proteins

- Access PIR ([pir.georgetown.edu](http://pir.georgetown.edu)) UniProt, iProClass and iProLink server Web pages for selected proteins in the spot list through their Swiss-Prot accession names.
- A two-step process enabled using the **(Edit | Select access to active DB server | ...)** checkbox command to select either SWISS-2DPAGE, UniProt, iProClass or iProLink servers.
- When you measure a spot and are connected to the Internet, it will also lookup the Swiss-Prot protein (accession name and protein id) on the SWISS-2DPAGE server.
- Then, if you enabled "Click to access DB", it will pop up the particular active PIR DB server you have selected.

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### Selecting Internet Proteome Reference Server



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### PIR UniProt Web Page for Identified Protein



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### PIR iProClass Web Page for Identified Protein



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### PIR iProLink Web Page for Identified Protein



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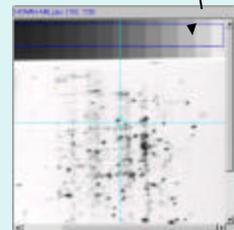
### Calibrating Grayscale for Better Quantification

- If the gel's stain/detection method is stoichiometric, then integrated density can correspond to protein concentration in a non-saturating range
- Exposure, the scanner and other systematic sources of non-linearity can be corrected to some degree by calibrating the image against a calibration standard
- Then map grayscale to that standard (e.g., optical density, CPM, etc.)
- Subsequent spot quantification will then be more accurate

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### Calibrating Grayscale with a ND Step-Wedge

1. The ND step wedge must be scanned with the image and the corresponding OD values known for each step
2. A region of interest (ROI) is overlaid on the step step-wedge
3. The ND wedge calibration wizard is invoked to analyze the data and estimate the calibration

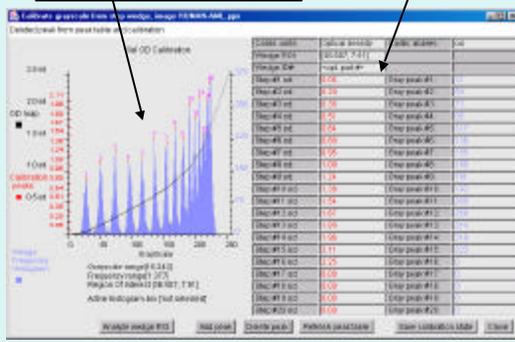


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## Calibrating Grayscale from ND Wedge Data

ROI histogram, peaks found and smooth extrapolated calibration curve

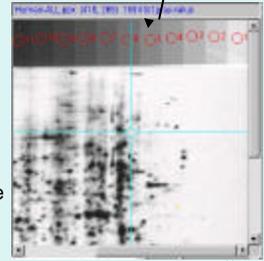
OD vs gray-peaks table



## Calibrating Grayscale with a Spot List of Calibrated Data

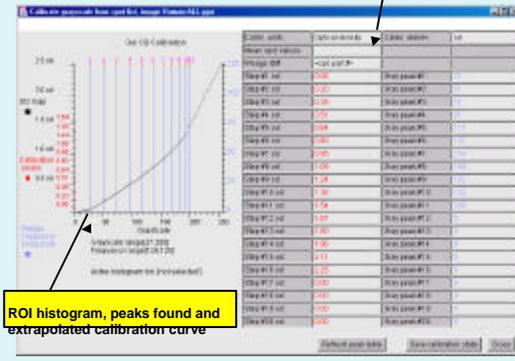
list of spots you defined

1. The image must contain calibrated regions with known concentrations or corresponding OD values known for each spot
2. You define a set of spots using (C-M) or (ALT-click)
3. The Spot List Calibration wizard is invoked to analyze the data and estimate the calibration



## Calibrating Grayscale from Spot List Data

OD vs gray-peaks table



ROI histogram, peaks found and extrapolated calibration curve

## Summary

- Flicker is an open-source 2D gel visual image comparison Java program freely available at <http://open2dprot.sourceforge.net/Flicker>
- Useful for visual comparison of 2D gels and other images
- Putative spot identification made by comparison with reference 2D gel databases
- Manual creation of spot-lists of estimated quantified spot densities can be exported (to Excel, etc.)

## References

- PDF and HTML documentation is available at: <http://open2dprot.sourceforge.net/Flicker>
- PF Lemkin, GC Thornwall, J Evans (2005) Comparing 2-D Electrophoretic Gels Across Internet Databases, in "The Proteomics Handbook", JM Walker (Ed), Humana Press Inc, Totowa, NJ, pp 279-305. <http://open2dprot.sourceforge.net/Flicker/PDF/Lemkin-ProteomicsProtocolsHandbook-279-306-2005.pdf>
- Full Web site and Reference Manual documentation: <http://open2dprot.sourceforge.net/Flicker/PDF/fullFikDoc.pdf>
- Early applet version: Lemkin PF (1997) Comparing Two-Dimensional Electrophoretic Gels across the Internet. Electrophoresis 18:461-470.

## Some Short-cut Menu Command Keys

- C-A add landmark after selecting both left and right image trial objects
- C-B capture background intensity value for current image under a circular mask
- C-D delete landmark - the last landmark defined
- C-E edit selected measured spot after clicking on spot to select it
- C-F toggle flickering the lower left and right images into the upper flicker window
- C-G toggle displaying gray values in the left and right image titles as move the cursor
- C-H show grayscale ROI histogram. Popup a histogram of the computation region ROI
- C-I Define or edit selected measured spot(s) annotation 'id' field
- C-J toggle spot measurement mode between list-of-spots and single measurements
- C-K delete selected measured spot after click on spot to select it
- C-L Define LRHC of region of interest (ROI) and assign to computing window
- C-M measure and show intensity under a circular mask for current image. Combine spot selection & measurement by holding **ALT-key** when press mouse to select spot.
- C-R measure and show intensity under computing window defined by the ROI
- C-T repeat the last Transform used, if one was previously performed else no-op
- C-U Define ULHC of the region of interest (ROI) and assign to computing window
- C-V Show data-window of selected pixel in the popup report window.
- C-W Clear the region of interest (ROI) and computing window
- C-Keypad "+" increase the canvas size for all three images
- C-Keypad "-" decrease the canvas size for all three images