History

Technology and Applications
Microscopy and Imaging

Quantimet 970 - 1983
Nanovid microscopy, VEDIC, 20 nm resolution, immunogold labeling - 1985

Pictures: Janssen Research Foundation

Automated Microscopy System

Imaging setup for stimulation studies of isolated cardiomyocytes in 1993
real-time acquisition (10 msec) and analysis.

Picture: Janssen Research Foundation
Imaging Software

SCIL Image 1.4 on Unix workstation – application development

Evolution of Imaging Platform

<table>
<thead>
<tr>
<th>Applications</th>
<th>Technologies</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImmunoGold labelling individual proteins</td>
<td>Nanovid ultra high resolution</td>
</tr>
<tr>
<td>Cardiomyocyte Ca²⁺ ratio / contraction</td>
<td>Automated acquisition</td>
</tr>
<tr>
<td>Neurite outgrowth</td>
<td>Parallel time lapse</td>
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<tr>
<td>Tissue Morphology</td>
<td>Distributed in-silico analysis</td>
</tr>
<tr>
<td>Cell morphology and Sub-cellular localization</td>
<td>Automatic sample detection</td>
</tr>
<tr>
<td>Model organism phenotypes</td>
<td>‘Unlimited’ mosaic size</td>
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<tr>
<td></td>
<td>Fast robust auto focusing</td>
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<td></td>
<td>Multi mode &amp; colour analysis</td>
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<tr>
<td></td>
<td>- Plate stacker</td>
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</table>
Current Status

Technology and Applications

Microscopy and Image Analysis

- Image Acquisition
  - Image acquisition
  - Image storage and retrieval
- Image Processing
  - Enhancement, restoration
  - Segmentation (objects)
- Object measurement
  - Feature extraction
  - Feature measurement
Image Acquisition

- Hardware
- Software
- Image Mosaics
- Time-lapse
- Autofocus system

Microscopy and Imaging Hardware

- Workstation for image acquisition control and image analysis
- Zeiss Axiocam.135 inverted microscope for cell biology
- Intensified camera for low-light, high-speed imaging

Cell Biology Setup

In Vivo Cell Biology Setup

Morphology Setup

Pictures: Janssen Research Foundation
Imaging Software
image capture, analysis & proofing

- position in the plate
- image in a tile
- actual image or analysis
- protocol settings
- progress status plate/stack

High Information Content Screening

Expression / Translocation
- Blue channel (nuclei)
- Green channel (ref)
- Red channel (TF)
- Pseudocolor overlay

Apoptosis
- Intact nuclei
- Fragmented nucleus
- Leaky membrane
- Fragmented nucleus

Pixel-matched images of 1 to 5 different emission wavelengths
Multiwell Plate Tiled Image Mosaic Scan

Automated tiled mosaic scanning of slides and several types of multi-well plates ranging from single wells to 1536 wells and more?

Tiled Pixel Matched Overlays

7x7 tile (Pseudocolor overlay)
### Multi Well Plate Scan

**Nuclear Localization Screening (NLS)**

- Hoechst stained nuclei of E11 synovial fibroblasts
  - 40x magnification, 60 wells
  - 4 x 5 tiled mosaic scanned
  - 693 x 513 pixels / image
  - 2772 x 2656 pix. / mosaic
  - Focus 1x in each well
  - 15 min. per plate x 2

[Image: Rectangular 4x5 image mosaic - view on one well]

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### Multi Well Plate Scan of C. elegans

- Central 60 wells of 96 well plate
  - 40x magnification (dry)
  - 473 512x512 pixel images / well
  - 20x focus / well (1200x / plate)
  - 28380 images in less than 4 hours
    - 0.5 seconds / image including travel
  - Mosaic of 12800 x 12800 pixels

[Image: Circular tiled mosaic scanned - view on one well]
Tissue Scan of Arbitrary Pattern

- Toluidin blue stained rabbit heart tissue, Epon 2 μ semi thin slice
  - 63x, immersion oil
  - 1300 512x512 images (0.75 Gb TIFF)
  - 433x auto focus
  - 40 minutes scan for complete mosaic
  - 51x66=3366 image tiles
  - 26112 x 33792 pixels (1.9 Gb)
  - Automated tissue edge detection by software

Tissue scan on ZEISS Axiophot
SONY 950P 3CCD RGB color camera

Pictures: Janssen Research Foundation

Video and Time-lapse Acquisition

- Time-related phenomena
  - Morphological changes
  - Motion analysis
- Multiple positions at once (max. 50 movies)
- Down to real-time (1/4 PAL video rate)

Time-lapse movie cultured PG12 (1994, courtesy R. Nuydens, PhD)

Video: Janssen Research Foundation
Robust Autofocus System - patented

- Need for robust general purpose autofocus system
- Fluorescence, bright field microscopy and phase contrast
- Reliable (>99%) at all magnifications (5x, ..., 40x, 63x)
- Insensitive to noise in fluorescence microscopy
- High speed, video rate implementation
- Large scale unattended operation possible

Robust Autofocusing in Microscopy, Jan-Mark Geusebroek et al., Cytometry 39:1-9 (2000)

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Autofocus and Scanning at Work

Video: Janssen Research Foundation
Image Processing

Management of Multi-Mode Tiles
Scale Space
Spatial Color Model

Multi-Mode Mosaic Framework - M³

- Automatic handling of large tiled image mosaics from multiple wells/positions.
- Manages structured image storage and retrieval
- Simultaneous analysis of up to 5 related tiled mosaics, e.g. for multi-mode (multiple channels) fluorescence microscopy.
- Analysis routine as a plug-in module.
- Short development cycle
- Embedded in applications
Scale Space

Differential Geometry
Gaussian Scale Space
Spatial Color Model

Conventional Image Processing
Filters and Thresholds

Threshold on gray value 128 - bright foreground versus dark background
The Visual System as a Geometry Engine

Geometry and scale

Analyzing Geometry

"edginess"  "curvedness"

Courtesy of Jan Koenderink
Differential Geometry
Shape and Scale

A 2D grayscale image landscape with gray value intensity seen as a 3D landscape

Geometric Patterns

Geometric patterns in an image
Describe with differential geometry
Differentiation with a Gaussian kernel

Differentiating a 2D image by convolution with the derivative of a Gaussian kernel

Gaussian Scale Selection

Intensity gradient Lw at scale (σ) 1, 5 and 10

camera noise elimination and size selection

Robust Autofocusing in Microscopy; J-M Geusebroek et al.,
Differential Geometry and Scale

Elliptic Patch Detection

- Detecting bright elliptic regions on a dark background
- Object size selection with $\sigma$ of Gaussian convolution kernel
- $\det \, H = |H_f| = f_{xx}f_{yy} - f_{xy}^2$
  - deviation of flatness: magnitude and direction
  - $f_{xx} < 0$ and $f_{xx}f_{yy} - f_{xy}^2 > 0$
  - $L_{xx} < 0$ and $L_{xx}L_{yy} - L_{xy}^2 > 0$
  - $L_{ww} < 0$ and $L_{vw}L_{ww} - L_{vw}^2 > 0$

Differential Geometry

Nuclear Localisation Screen

Processing noisy images

$L_{xx} < -0.0004$ and $L_{xx}L_{yy} - L_{xy}^2 > 0$; scale sigma ($\sigma$) 9.0

40x, Hoechst stained nuclei; Photonic Science ISIS-3 intensified CCD camera
### Differential Geometry

#### Line detection in Neurite Tracking

A line detector for dark ridges on an uneven background

\[ L_{xx}L_{yy} - 2L_{xy}L_{xy} + L_{yy}L_{xx} \text{ or } L_{pp} > 4.0 \times \text{threshold} / \sigma^2 \]

Scale (\(\sigma\)) 2.0, threshold 1.0

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**Pictures:** Janssen Research Foundation

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### Differential Geometry - Spirochetes

Spirochetes are seen with a Warthin-Starry silver stain.

\[ \text{inv_ridges, scale (} \sigma \text{) 1.0, dark ridges} \]
Differential Geometry - Myelin

Line detection on myelinated axon myelin sheats.

Sample preparation:
Toluidin blue stained, 1 µm Epon embedded sections

Scanning:
40x immersion oil, autofocus every 3 images

A line detector for dark ridges:
$L_{xx} + L_{yy} - 0.5*\sqrt{(L_{xx}^2 + 4L_{xy}^2)} > 0$

Gaussian scale ($\sigma$) 1.5

Spatial Color Model

Object Properties
Color Perception and Color Models
Combining Color and Spatial Extent
Robustness in applications
Visible Light as Electromagnetic Radiation

The visible light region consists of a spectrum of wavelengths, which range from approximately 700 nanometers to approximately 400 nm; that would be 7 \times 10^{-7} m to 4 \times 10^{-7} m.

The Photometric Reflection Model and Color Invariance

The influence of the light source, object shading and highlights, the object’s shape (geometric information) and reflectance properties (photometric information), Kubelka-Munk theory describing the optical property of a turbid medium which absorbs and scatters light, Fresnel reflectance of smooth surfaces, Lambertian reflection or diffuse reflection, Shafte's dichromatic reflection model.


Courtesy of Jan-Mark Geusebroek
Photometric reflection model

\[ E(\lambda, x) = m(x)l(\lambda, x)c(\lambda, x) \]

assumption:
\[ l(\lambda, x) = l(\lambda) \]

differentiation (\( E = m \cdot l \cdot c \)):

\[
\frac{\partial E}{\partial \lambda} = mc \frac{\partial l}{\partial \lambda} + ml \frac{\partial c}{\partial \lambda}
\]

\[
\frac{1}{E} \frac{\partial E}{\partial \lambda} = \frac{1}{l} \frac{\partial l}{\partial \lambda} + \frac{1}{c} \frac{\partial c}{\partial \lambda}
\]

and thus:

\[
\frac{\partial}{\partial x} \left( \frac{1}{E} \frac{\partial E}{\partial \lambda} \right) = \frac{1}{c} \frac{\partial^2 c}{\partial \lambda \partial x} - \frac{1}{c^2} \frac{\partial c}{\partial \lambda} \frac{\partial c}{\partial x} - \frac{1}{E^2} \frac{\partial |E|}{\partial x} \]

Object Reflectance Edge Detector

\[
\frac{\partial}{\partial x} \left( \frac{1}{E} \frac{\partial E}{\partial \lambda} \right) = \frac{\partial}{\partial x} \left( \frac{E_\lambda}{E} \right) = \frac{E_{\lambda x} E - E_\lambda E_x}{E^2}
\]
Hierarchy of Invariants

<table>
<thead>
<tr>
<th>Illumination Intensity</th>
<th>Shadow</th>
<th>Highlights</th>
<th>Illumination Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>N</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>W</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

• H is related to the Hue
• N describes object reflectance independent of the illumination
• C describes object color regardless of intensity
• W determines changes in object reflectance independent of illumination intensity

Hierarchy of invariants within the Kubelka-Munk model:
\[ H \subseteq N \subseteq C \subseteq W \subseteq E \]

Invariant Edge Detectors

- Detecting color edges (gradient magnitude) with increasing invariance for shading and highlights and finally only the object properties.
CIELAB Color Model

Lab in the CIELAB color model refer to analogs of the three Hering opponent process dimensions. 
$L^*$ is the luminosity dimension, $a^*$ is the red-green contrast, $b^*$ is the yellow-blue contrast.

Sensitivity of the Human Visual System

The cones in the retina are the fundamental units of visual information.
Long (R: 565 nm) Medium (G: 530 nm) and Short (B: 435 nm) Wavelengths.

Courtesy of Jan-Mark Geusebroek
Trichromatic and Opponent Color Theory

Ewald Hering, Zur Lehre vom Lichtsinn, Vienna 1878

The Red Green and Blue sensitivity curves are not orthogonal. Orthogonalization leads to the graphs shown here.
The visible spectrum (wavelength $\lambda$) is probed with a Gaussian kernel $\lambda_g$, centered on 520 nm and width $\sigma_g = 55.0$. 

Spectral measurement by a physical device without knowledge about the environment

Courtesy of Jan-Mark Geusebroek
Combining Color and Space

Color differentiation up to the second order, combined with Gaussian Scale Space
integration over spectral and spatial dimensions.

Color Intensity Invariance

Detection of color transitions in images.

\[ E_\lambda > 0, \text{ zero crossing intensity invariance} \]

\[ \text{intra- and inter scene illumination intensity changes} \]
Spatial Color Model - Feulgen stain

Paramecium caudatum, Feulgen and Fast green stain
Color canny, red-green normalized edges, scale ($\sigma$) 3

Spatial Color Model and Tracing Color Edges

An example of color invariant edge detection. Influence of illumination color temperature on edge strength, scale ($\sigma$) is 3.0. Skin tissue section illuminated by a halogen bulb at 4000K (top) and 2600K (bottom) color temperature.

Pictures: Janssen Research Foundation
Spatial Color Model in Fluorescence Microscopy

Red: $E_\lambda > 0$, $E_{\lambda\lambda} > 0$, $E_\lambda - E_{\lambda\lambda} < 0$
Green: $E_\lambda > 0$, $E_{\lambda\lambda} < 0$
Blue: $E_\lambda < 0$, $E_{\lambda\lambda} > 0$, $E_\lambda - E_{\lambda\lambda} < 0$
Orange: $E_\lambda > 0$, $E_{\lambda\lambda} > 0$, $E_\lambda - E_{\lambda\lambda} > 0$
Scale ($\sigma$) 1.0

TetraSpeck 4.0 µm beads photographed using optical filter sets appropriate for DAPI, fluorescein, rhodamine and Texas Red dye.

Spatial Color Model – Hematoxylin eosin stain

Pituitary gland, sheep, adenohypophysis 40x
Cell: $E_\lambda < 0$, $E_{\lambda\lambda} > 0$, scale $\sigma$ 1.0
Nuclei: $E_\lambda < 0$, $E_{\lambda\lambda} > 0$, $E_\lambda + E_{\lambda\lambda} < 0$, scale $\sigma$ 3.0
additional constraint added to refine selection
Spatial Color Model - Blood Smear

Blood smear, Giemsa stain, 100x
JPEG compression

RBC: $E_\lambda > 0$, $E_\lambda + E_{\lambda\lambda} > 0$, scale ($\sigma$) 0.5
Leucocytes: $E_\lambda < 0$, scale ($\sigma$) 12
Leucocyte nuclei: $E_\lambda < 0$, $E_{\lambda\lambda} > 0$, scale ($\sigma$) 3

Spatial Color Model - PAS stain

$L_{ww} > 0$, $L_{vvL_{ww}} - L_{vw}^2 > 0$, $E_{\lambda\lambda} - E_\lambda > 0$
Scale ($\sigma$) 2.0

P.A.S. stain for carbohydrates (goblet cells, gut)
carbohydrates stain magenta – elliptic patches

Pictures: Janssen Research Foundation

Object Measurement and Presentation

Tabulated data
Export for statistical analysis

Measurement Data Report

<table>
<thead>
<tr>
<th>Object</th>
<th>Area</th>
<th>Perimeter</th>
<th>Contour ratio</th>
<th>Grey mean</th>
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<tbody>
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<td>69.65</td>
<td>1.22</td>
<td>109.65</td>
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<td>200.04</td>
</tr>
</tbody>
</table>

Detailed density and shape measurements, export to S-Plus, SAS, Excel, ....
Detailed data export
Nuclear Expression Readout & data analysis

**M³ Imaging Framework**

- Designed for pharmaceutical and biotechnology research
  - From (Sub)Cellular assays and tissue morphology to model organisms
  - Living cells and fixed tissue
  - Fluorescence, brightfield microscopy, phase contrast, ...
- Automated image acquisition
  - High speed and precision motorized microscope stage control
  - High magnification (5x, ..., 40x, 63x oil immersion) tiled image mosaics
  - Robust real-time automatic focus algorithm
  - Automatic calibration
  - Time-lapse movies in parallel
- Image analysis
  - General purpose framework for Multi-Mode Mosaic microscopy (M³)
  - Sophisticated structural algorithms (differential geometry, mathematical morphology)
  - Color based mathematical analysis (spatial color model)
  - Distributed computing power (Unix workstations and servers)
## Summary

**Industrial biological imaging platform:**
- Fast and robust automated image acquisition
  - Flexible acquisition platform
  - Enabling low light - high resolution applications
  - Viable sample conditions
- Powerful automated image analysis
  - Robust & sensitive object recognition algorithms
  - Parallel multiple feature measurement
  - Scalable computing platform

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### References


