UNIVERSITY OF BOLOGNA – ARCES
European Doctorate in Information Technology, XXV cycle

SOLUTIONS TO COMMON ISSUES IN WIDEFIELD MICROSCOPY: VIGNETTING, MOSAICING AND DEPTH OF FOCUS
Filippo Piccinini

Coordinator
Prof. Claudio Fiegna

Tutor
Prof. Alessandro Bevilacqua

Co-tutor
Prof. Mauro Ursino

Doctor Europaeus: January 1, 2010 - December 31, 2012
Final exam: April 19, 2013

Overview on the Doctorate

MOSAICING

BIOMEDICAL ENGINEERING
Overview on the Doctorate

MOSAICING

LIGHT MICROSCOPY

HISTOLOGICAL SAMPLE (2D) ➔ VIGNETTING

CELL CULTURE (2D) ➔ VIGNETTING
Overview on the Doctorate

MOSAICING

LIGHT MICROSCOPY

HISTOLOGICAL SAMPLE (2D)

CELL CULTURE (2D)

VIGNETTING

VIGNETTING

ADVANCE
In partnership with Osteoarticular Regeneration Laboratory, Rizzoli Orthopaedic Institute, IOR, Bologna

Automatic non-invasive system based on high content analysis to detect and characterize ViAl mesenchimal stem Cells in a spatio-temporal contExt (ADVANCE)

ADVANCE
In partnership with Osteoarticular Regeneration Laboratory, Rizzoli Orthopaedic Institute, IOR, Bologna

STAMINAL
In partnership with Laboratory of Radiobiology, Istituto Romagnolo per lo Studio e la cura dei Tumori, IRCCS-IRST, Meldola

Characterization of Stem cells by means of AutoMatic ANALysis of microscopic images in pre-clinic therapy (STAMINAL)
Overview on the Doctorate

**MOSAICING**

LIGHT MICROSCOPY

HISTOLOGICAL SAMPLE (2D)

CELL CULTURE (2D and 3D)

**VIGNETTING**

**DEPTH-OF-FOCUS**

FLUORESCENCE MICROSCOPY

**VIGNETTING**

ADVANCE
In partnership with Osteoarticular Regeneration Laboratory, Rizzoli Orthopaedic Institute, IOR, Bologna

STAMINAL
In partnership with Laboratory of Radiobiology, Istituto Romagnolo per lo Studio e la cura dei Tumori, IRCCS-IRST, Meldola

VIGHCS
In partnership with Light Microscopy and Screening Center, Eidgenossische Technische Hochschule Zurich, ETHZ, Zurich, Switzerland.
Overview on the Doctorate

<table>
<thead>
<tr>
<th>PROJECTS - ORGANIZATION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2010</strong></td>
</tr>
<tr>
<td>Winter</td>
</tr>
<tr>
<td>ADVANCE</td>
</tr>
</tbody>
</table>

**ADVANCE**
in partnership with Osteoarticular Regeneration Laboratory, Rizzoli Orthopaedic Institute, IOR, Bologna

**STAMINAL**
in partnership with Laboratory of Radiobiology, Istituto Romagnolo per lo Studio e la cura dei Tumori, IRCCS-IRST, Meldola

**VIGHCS**
in partnership with Light Microscopy and Screening Center Eidgenossische Technische Hochschule Zurich, ETHZ, Zurich, Switzerland

Outline

- Vignetting in light microscopy (**ADVANCE**)
- Vignetting in fluorescence microscopy (**VIGHCS**)
- Mosaicing techniques (**ADVANCE**)
- Reconstruction from depth-of-focus (**STAMINAL**)
- Brief summary of the Doctorate
Vignetting

EARLY PROBLEM THAT AFFECTS DIGITAL IMAGING
Fall-off of brightness intensity from the principal point towards the image borders due to an uneven illumination field, lens, etc

IF NO CORRECTION IS ACCOMPLISHED, THE IMAGES COULD BE NOT COMPARABLE

Flat-field correction

\[ I_{FFC} = \frac{I_{ORI}}{V} \]

Vignetting correction by exploiting an optic:

Mathematically, vignetting is one of the most common problems that affect digital imaging. The effect shows up as a characteristic fall-off of brightness intensity from the principal point towards the image borders due to an uneven illumination field, lens, etc. If no correction is accomplished, the images could be not comparable. Flat-field correction is a technique that corrects the vignetting effect. The corrected image intensity is proportional to the original image intensity, where the correction factor is a constant factor. The method to produce uniformity in the image can be mathematically modeled using the flat-field correction technique.
State of the art: common approach

Vignetting function estimated using

**HOMOGENEOUS REFERENCE FREE OF OBJECTS**

- Empty field (in light microscopy)
- Fluorescence calibration slide (in fluorescence microscopy)

*SEVERAL REASONS COULD MAKE THIS APPROACH DIFFICULT TO APPLY*

Background in light microscopy

**CELL CULTURES**
**HISTOLOGICAL SAMPLES**
**BRIGHTFIELD**
**PHASE-CONTRAST**

- Cover the most relevant part of the biological routine examinations
- Most common imaging techniques in light microscopy

**BACKGROUND**
Culture’s medium for cell cultures, glass slide for histological sample
Considered uniform and of optical properties quite similar to the foreground
Our approach

Vignetting function estimated using IMAGES’ BACKGROUND

From an image made of background it is possible reconstructing the vignetting function
Our approach

1. Images Acquisition
2. Background Segmentation
3. Vignetting Reconstruction

DERIVATIVE MASK
ADAPTIVE THRESHOLDING
MORPHOLOGICAL OPENING
SMALL AREAS DELETION
Our approach

1. Images Acquisition
2. Background Segmentation
3. Vignetting Reconstruction

- Derivative Mask
- Adaptive Thresholding
- Morphological Opening
- Small Areas Deletion
Our approach

- Images Acquisition
- Background Segmentation
- Vignetting Reconstruction

- Derivative Mask
- Adaptive Thresholding
- Morphological Opening
- Small Areas Deletion
- Z-Median Filtering
- Holes Filling
Some of the experiments performed

- Comparison of the shape of different vignetting functions
- Effectiveness of image correction using different vignetting functions
- Numerical analysis of flat field corrections using mosaics
Some of the experiments performed

Starting from real stacks of images regarding cell cultures and histological specimens, we compared the vignetting function estimated from empty field (typically used in literature as the ground truth) and the vignetting function estimated from:

- reference images regarding glass slide or culture medium only, free of cells
- the stacks themselves with the proposed method

Absolute mean difference < 7%

Some of the experiments performed

- Comparison of the shape of different vignetting functions
- Effectiveness of image correction using different vignetting functions
- Numerical analysis of flat field corrections using mosaics
Some of the experiments performed

Our method is resulted always the best or the second
Results always better than using Empty Field

<table>
<thead>
<tr>
<th>flatness</th>
<th>image 1</th>
<th>image 2</th>
<th>image 3</th>
<th>image 4</th>
<th>image 5</th>
<th>image 6</th>
<th>image 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO CORRECTION</td>
<td>1.91</td>
<td>1.80</td>
<td>1.35</td>
<td>1.70</td>
<td>1.62</td>
<td>1.55</td>
<td>1.79</td>
</tr>
<tr>
<td>EMPTY FIELD</td>
<td>1.13</td>
<td>0.93</td>
<td>0.99</td>
<td>0.84</td>
<td>0.87</td>
<td>0.87</td>
<td>0.86</td>
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<tr>
<td>GLASS SLIDE</td>
<td>1.12</td>
<td>0.89</td>
<td>0.93</td>
<td>0.80</td>
<td>0.83</td>
<td>0.75</td>
<td>0.83</td>
</tr>
<tr>
<td>CULTURE MEDIUM</td>
<td>0.65</td>
<td>0.67</td>
<td>0.75</td>
<td>0.50</td>
<td>0.61</td>
<td>0.69</td>
<td>0.65</td>
</tr>
<tr>
<td>OUR METHOD</td>
<td>0.56</td>
<td>0.54</td>
<td>0.83</td>
<td>0.55</td>
<td>0.54</td>
<td>0.71</td>
<td>0.53</td>
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Some of the experiments performed

- Comparison of the shape of different vignetting functions
- Effectiveness of image correction using different vignetting functions
- Numerical analysis of flat field corrections using mosaics
Some of the experiments performed

Our method achieved the flattest mosaics

<table>
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<tr>
<th>VIGNETTING FUNCTION</th>
<th>RMSE</th>
<th>SNR</th>
<th>UQI</th>
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<tbody>
<tr>
<td>NO CORRECTION</td>
<td>4.26</td>
<td>29.31</td>
<td>0.7469</td>
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<tr>
<td>EMPTY FIELD</td>
<td>2.88</td>
<td>32.78</td>
<td>0.8606</td>
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<tr>
<td>GLASS SLIDE</td>
<td>2.90</td>
<td>32.70</td>
<td>0.8587</td>
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<tr>
<td>CULTURE MEDIUM</td>
<td>2.96</td>
<td>32.50</td>
<td>0.8544</td>
</tr>
<tr>
<td>OUR METHOD</td>
<td>2.77</td>
<td>33.09</td>
<td>0.8691</td>
</tr>
</tbody>
</table>

Vignetting in light microscopy

CONCLUSIONS

- We proposed a method to estimate the vignetting function from the images themselves without requiring prior information
- We proved that the results achieved with the proposed method are better, or at least comparable, than those achievable using the vignetting function estimated from empty field (typically used as the ground truth)
Publications


A. Bevilacqua, F. Piccinini, "Is an empty field the best reference to correct vignetting in microscopy?", 7th International Workshop on Biosignal Interpretation (BSI), Como, Italy, July 2-4, 2012, pp. 267-270.


Outline

• Vignetting in light microscopy (ADVANCE)

• Vignetting in fluorescence microscopy (VIGHCS)

• Mosaicing techniques (ADVANCE)

• Reconstruction from depth-of-focus (STAMINAL)

• Brief summary of the Doctorate
Stays at ETH Zurich

2010
CIMST 2010 Interdisciplinary Summer School on Bio-medical Imaging
ETH Zurich, Switzerland, September 6-17, 2010

2011
3-month stay at the Light Microscopy and Screening Centre
ETH Zurich, Switzerland, May 9 - August 26, 2011

2012
3-month stay at the Light Microscopy and Screening Centre
ETH Zurich, Switzerland, May 7 - August 8, 2011

HCS - High Content Screening

LMSC - Light Microscopy and Screening Centre
RISC - RNA Image-based Screening Centre
Dr. Peter Horvath, Image and Data Analysis

HSC to extract quantitative data from complex biological systems with subcellular resolution

TO CORRECT THE IMAGES BY VIGNETTING IS FUNDAMENTAL TO PERFORM RELIABLE MEASUREMENTS
Fluorescence microscopy in HCS

- BACKGROUND BLACK
- BACKGROUND AND FOREGROUND DISTRIBUTION DIFFERENT
- AUTOMATIC ROBOTIZED SYSTEM
- MANY IMAGES AT ONE’S DISPOSAL
LCBM - Linear Correction Based Method

**Background Reconstruction**

**Images Acquisition**

**Vignetting Reconstruction**

**Stack Building**

**Z-Histogram Analysis**
LCBM - Linear Correction Based Method

**BACKGROUND**
- Reconstruction
- Vignetting
- Images

**ACQUISITION**
- Stack Building
- Z-Histogram Analysis
- Background Modeling

**HISTOGRAM ANALYSIS**
- Grey levels
- Frequency
LCBM - Linear Correction Based Method

Images Acquisition → Background Reconstruction → Vignetting Reconstruction

Stack Building
Z-Histogram Analysis
Background Modeling

LCBM - Linear Correction Based Method

Images Acquisition → Background Reconstruction → Vignetting Reconstruction

Stack Building
Z-Histogram Analysis
Background Modeling
Foreground Modeling
LCBM - Linear Correction Based Method

- **IMAGES ACQUISITION**
- **BACKGROUND RECONSTRUCTION**
- **VIGNETTING RECONSTRUCTION**

- **STACK BUILDING**
- **Z-HISTOGRAM ANALYSIS**
- **BACKGROUND MODELING**
- **FOREGROUND MODELING**

Fluorescence microscopy in HCS

- **BACKGROUND BLACK**
- **BACKGROUND AND FOREGROUND DISTRIBUTION DIFFERENT**
- **AUTOMATIC ROBOTIZED SYSTEM**
- **MANY IMAGES AT ONE’S DISPOSAL**
nLCBM - non Linear Correction Based Method

Starting from ideas regarding outdoor images

VIGNETTING FROM OVERLAPPING IMAGES

VIGNETTING FROM OVERLAPPING IMAGES

CONCEPT

Vignetting function constant → reconstructed by imaging the same object in different positions
Vignetting estimation, crucial steps

- PHOTO-BLEACHING CORRECTION

- IMAGE REGISTRATION
Vignetting estimation, crucial steps

- PHOTO-BLEACHING CORRECTION
- IMAGE REGISTRATION
- INTENSITY QUANTIZATION

THIN PLATE SPLINE (Duchon, 1976)
non-parametric fitting method with closed-form solution for estimating the 2D surface minimizing the integral of the squared second derivative of a specific energy function.

SPLINE
smooth polynomial function piecewise-defined. It possesses a high degree of smoothness at the places where the polynomial pieces connect.
Vignetting estimation

SET OF VIGNETTING FUNCTIONS

Methods compared

**METHOD 1: Fluorescence Calibration Slide (FCS)**
- Vignetting function: image from fluorescence calibration slide
- Background: image from region free of objects
- flat-field correction:
  \[
  I_{FCC} = \frac{I_{cal} - B}{V + B}
  \]

**METHOD 2: CellProfiler “mean” (CPmean)**
- Vignetting function: z-mean intensities
- flat-field correction:
  \[
  I_{FCC} = \frac{I_{cal}}{V}
  \]

**METHOD 3: CellProfiler “mean” and “background” (CPboth)**
- Vignetting function: z-mean intensities
- Background: z-minimum intensities resizing the images
- flat-field correction:
  \[
  I_{FCC} = \frac{I_{cal} - B}{V - B} + \frac{B}{V - B}
  \]
Some of the experiments performed

**EXP 1:** flatness of the median foreground surfaces computed using 500 images flat-field corrected according to the different tested methods

*flatness:* standard deviation (σ) of the local mean values of the surface

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<tr>
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<tr>
<td><strong>flatness</strong></td>
<td>3.33</td>
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<td>2.21</td>
<td>2.37</td>
<td>1.75</td>
<td>1.81</td>
</tr>
<tr>
<td><strong>simple σ</strong></td>
<td>4.22</td>
<td>4.41</td>
<td>3.59</td>
<td>3.74</td>
<td>3.05</td>
<td>3.15</td>
</tr>
<tr>
<td><strong>RANK</strong></td>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
<td>6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
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<td>4th</td>
<td>1st</td>
<td>2nd</td>
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Some of the experiments performed

**EXP 2:** intensity difference (Root Mean Squared Error) computed in the overlapping of 100 image pairs flat-field corrected according to the different tested methods
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Some of the experiments performed.
Some of the experiments performed

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<tr>
<td>RMSE ($\mu\sigma$)</td>
<td>15.41±3.82</td>
<td>13.14±3.64</td>
<td>13.15±3.45</td>
<td>13.05±3.58</td>
<td>12.85±3.60</td>
<td>12.98±3.60</td>
</tr>
<tr>
<td>Average improvement</td>
<td>-</td>
<td>15.14%</td>
<td>14.12%</td>
<td>15.68%</td>
<td>17.07%</td>
<td>16.09%</td>
</tr>
<tr>
<td>RANK</td>
<td>6th</td>
<td>4th</td>
<td>5th</td>
<td>3rd</td>
<td>1st</td>
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</table>
CONCLUSIONS

- This is the first work where the vignetting is tackled as a non-linear problem.
- Both the methods we proposed perform better than the other methods typically used in HCS.
- We proved that using Fluorescent Calibration Slide for correcting vignetting could also worsen the distribution of the signal in the images.

PUBLICATIONS

F. Piccinini, A. Bevilacqua, K. Smith, P. Horvath, “Vignetting and photo-bleaching correction in automated fluorescence microscopy from an array of overlapping images”, 10th International Symposium on Biomedical Imaging (ISBI), San Francisco, CA, USA, April 7-11, 2013.


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- Brief summary of the Doctorate
Mosaic

A large image obtained stitching together two or more images without losing resolution

REASONS OF MOSAICING

- Seeing a global condition of objects
- Storing an instantaneous state of objects in contest
- Capturing large objects of interest with a high resolution
- Performing time lapse experiments
- Performing high content analysis
- Supporting the operator during the navigation
- Acquiring information of unexpected (tissue, cells) objects during a normal inspection
Mosaicing

**GOALS**

- Testing different vignetting correction approaches
- Comparing different registration approaches

**MicroMos**

- Mosaicing tool for obtaining on-line mosaics of image acquired using widefield microscopes


Mosaicing – on-line method

**FEATURES DETECTION AND MATCHING**  **WARPING MODEL ESTIMATION**  **IMAGE WARping AND STITCHING**
Mosaicing – on-line method

Shi-Tomasi corners, Lucas-Kanade tracker

Original image

**Mosaicing – on-line method**

**FEATURES DETECTION AND MATCHING** → **WARPING MODEL ESTIMATION** → **IMAGE WARPING AND STITCHING**

**TRANSLATIVE**

\[
H = \begin{bmatrix}
1 & 0 & t_1 \\
0 & 1 & t_2 \\
0 & 0 & 1
\end{bmatrix}
\]

**AFFINE**

\[
H = \begin{bmatrix}
 h_{11} & h_{12} & t_1 \\
h_{21} & h_{22} & t_2 \\
 0 & 0 & 1
\end{bmatrix}
\]

**PROJECTIVE**

\[
H = \begin{bmatrix}
r_{11} & r_{12} & t_1 \\
r_{21} & r_{22} & t_2 \\
 h_{31} & h_{32} & 1
\end{bmatrix}
\]

One corresponding point

Three corresponding points

Four corresponding points
Mosaicing – on-line method

<table>
<thead>
<tr>
<th>FEATURES DETECTION AND MATCHING</th>
<th>WARPING MODEL ESTIMATION</th>
<th>IMAGE WARPING AND STITCHING</th>
</tr>
</thead>
</table>

### FRAME-TO-FRAME (F2F)

\[
H_{Tn}^{0} = \prod_{i=1}^{n} H_{Ti}^{(i-1)} \\
I_{0} = H_{Tn}^{0} \cdot I_{n}
\]

### FRAME-TO-MOSAIC (F2M)

\[
B_{n} = M_{I(n-1)}^{0} \cdot H_{I_{n}}^{(n-1)} \cdot I_{n} \\
M_{I_{n}}^{0} = \prod_{i=1}^{n} H_{I_{i}}^{(i-1)} \cdot H_{B_{i}}^{S_{i}} \\
I_{0} = M_{I_{n}}^{0} \cdot I_{n}
\]
Mosaicing – on-line method

- VIGNETTING CORRECTION
- FEATURES DETECTION AND MATCHING
- WARPING MODEL ESTIMATION
- IMAGE WARPING AND STITCHING

30 Mesenchymal Stem Cells images
Some of the experiments performed

**TONAL REGISTRATION**
- No tonal registration
- Vignetting correction

**WARPING MODEL**
- Translative
- Affine
- Projective

**GEOMETRIC REGISTRATION**
- F2F
- F2M

**JOINT TONAL AND GEOMETRIC REGISTRATION ERROR**
RMSE between Back-Projected (BP) images and Overlapped Parts (OP) of the mosaic

\[ RMSE_n = \sqrt{\frac{\sum_x \sum_y [OP_n(x, y) - BP_n(x, y)]^2}{P}} \]

**GEOMETRIC REGISTRATION ERROR ONLY**
Normalized Euclidean Norm (NEN) between Global Matrix (GM, estimated using looping-path mosaics) and Identity Matrix (IM)

\[ NEN = \sqrt{\sum_{e=1}^{E} (GM(e) - IM(e))^2} \]
Some of the experiments performed

MOSAICS OF 30 MESENCHYMAL STEM CELLS IMAGES

<table>
<thead>
<tr>
<th>Algorithm’s configuration</th>
<th>Model</th>
<th>Translative</th>
<th>Affine</th>
<th>Projective</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: F2M with tonal registration</td>
<td></td>
<td>2.67 ± 0.98</td>
<td>2.05 ± 0.20</td>
<td>2.02 ± 0.16</td>
</tr>
<tr>
<td>2: F2F with tonal registration</td>
<td></td>
<td>3.49 ± 1.34</td>
<td>2.13 ± 0.25</td>
<td>2.12 ± 0.28</td>
</tr>
<tr>
<td>3: F2M without tonal registration</td>
<td></td>
<td>4.43 ± 0.85</td>
<td>4.31 ± 1.23</td>
<td>4.30 ± 1.19</td>
</tr>
<tr>
<td>4: F2F without tonal registration</td>
<td></td>
<td>4.96 ± 1.31</td>
<td>4.18 ± 0.76</td>
<td>4.29 ± 0.95</td>
</tr>
</tbody>
</table>
Some of the experiments performed

### MOSAICS OF 30 MESENCHYMAL STEM CELLS IMAGES

#### RMSE ($\mu \pm \sigma$)

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<td>2: F2F with tonal registration</td>
<td></td>
<td>1.68 ± 0.76</td>
</tr>
<tr>
<td>3: F2M without tonal registration</td>
<td></td>
<td>1.07 ± 0.45</td>
</tr>
<tr>
<td>4: F2F without tonal registration</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSIONS

- We developed a user-friendly tool to obtain on-line mosaics of images acquired using a widefield microscope, also testing different registration configurations.
- The tonal registration always provides improvements.
- Generally, in widefield microscopy the best mosaicing warping model is the projective one, although the translative model is often used.

PUBLICATIONS


Outline

- Vignetting in light microscopy (ADVANCE)
- Vignetting in fluorescence microscopy (VIGHCS)
- Mosaicing techniques (ADVANCE)
- Reconstruction from depth-of-focus (STAMINAL)
- Brief summary of the Doctorate
Depth-of-focus

**PROBLEM**

With a standard objective, it is impossible to acquire a single completely sharp image of objects characterized by a large depth or of a set of objects placed at different focus planes.

**SPHEROID**: large dimension multi-cellular aggregate obtained *in-vitro* starting from a single cancer cell taken directly from patient's tumor. The spheroids represent a solid 3D tumor model to test and compare different radiotherapy treatments.
Depth-of-focus

DEPTH-OF-FOCUS (DF)
Distance between focal planes in the z-dimension, where objects keep sharp or in-focus.

OBJECT RECONSTRUCTION
Starting from a set of images acquired at different depths (z-plane), if the z-distance between two subsequent images is less than DF, it is possible to reconstruct a single 2D image completely sharp where each part of the objects is in-focus.
Starting from a set of images acquired at different depths (z-plane), if the z distance between two subsequent images is less than DF, it is possible to reconstruct a single 2D image completely sharp where each part of the objects is in-focus.
Depth-of-focus

OBJECT RECONSTRUCTION
Starting from a set of images acquired at different depths (z-plane), if the z distance between two subsequent images is less than DF, it is possible to reconstruct a single 2D image completely sharp where each part of the objects is in-focus.
State of the art

Many different methods have been proposed in literature to extend the microscopes’ depth of focus

- STACK FOCUSER (SF)
- DEPTH FROM FOCUS (DFF)
- COMPLEX WAVELET-BASED METHOD (CWBM)
- MODEL-BASED METHOD (MBM)
- CURVELET METHOD (CUR)

PROBLEM
There is not a standard metric for comparing the different methods

Depth-of-focus

OUR GOALS

- TO VALIDATE A METRIC FOR COMPARING THE DIFFERENT METHODS **
- TO DEVELOP A BETTER METHOD FOR EXTENDING THE DEPTH OF FOCUS II
Depth-of-focus

OUR GOALS

- TO VALIDATE A METRIC FOR COMPARING THE DIFFERENT METHODS

- TO DEVELOP A BETTER METHOD FOR EXTENDING THE DEPTH OF FOCUS


Metric for comparing the methods

METRIC REQUIRING THE GROUND TRUTH

Universal Quality Index (UQI) computed using the output 2D image ($I$) completely in focus obtained by the different methods tested and the ground truth ($G$)

\[
UQI_{(G,I)} = \left( \frac{\mu(I) \cdot \mu(I)}{\sigma^2_G + \mu^2_I} \right) \cdot \left( \frac{2 \cdot \mu_G \cdot \mu_I}{\sigma^2_G + \mu^2_I} \right) \cdot \left( \frac{2 \cdot \sigma_G \cdot \sigma_I}{\sigma^2_G + \sigma^2_I} \right)
\]

SYNTHETIC DATA SETS CORRELATED OF GROUND TRUTH
Metric for comparing the methods

**METRICS NOT REQUIRING THE GROUND TRUTH**


Using synthetic data sets correlated of ground truth we proved that these metrics are not able to rank the method as the UQI does.

**NEW TWO STEPS EVALUATION APPROACH**

- Discarding methods clearly yielding visually poor results
- Computing the Average UQI (AUQI) between the output 2D image (I) completely in focus obtained by the different methods tested and the N slices (Sn) acquired at different depths

\[
AUQI(S_1, \ldots, S_N, I) = \frac{1}{N} \sum_{n=1}^{N} UQI(S_n, I)
\]

**Depth-of-focus**

**OUR GOALS**

- TO VALIDATE A METRIC FOR COMPARING THE DIFFERENT METHODS **
- TO DEVELOP A BETTER METHOD FOR EXTENDING THE DEPTH OF FOCUS II

**Depth Of Focus Extender (DOFE)**

**METHOD**

While an object's region is in focus, it is sharp and well defined. When the focus is lost the region becomes smooth. Starting from a stack of images of the same object, but acquired at different plane's depth (z-plane), for every region, we can search in the stack the maximum of derivatives to understand which image expresses the best focus.

**ALGORITHM PIPELINE**

- Stack acquisition
- Edge detection
- Derivative filtering
- Depth map creation
- Depth map filtering
- Composite image creation
## Depth-of-focus

<table>
<thead>
<tr>
<th>INPUT IMAGES STACK</th>
<th>OUTPUT DOFE</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Input Image" /></td>
<td><img src="image2.png" alt="Output Image" /></td>
</tr>
</tbody>
</table>

## Depth-of-focus

<table>
<thead>
<tr>
<th>OUTPUT STACK FOCUSER</th>
<th>OUTPUT DOFE</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image3.png" alt="Stack Image" /></td>
<td><img src="image4.png" alt="Output Image" /></td>
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</table>
Depth-of-focus

OUTPUT CWBM
OUTPUT DOFE

Some of the experiments performed

SYNTHETIC DATA SETS WITH GROUND TRUTH

Brodatz textures

METRIC UQI

<table>
<thead>
<tr>
<th>UQI, SET</th>
<th>DOFE</th>
<th>SF</th>
<th>DFF</th>
<th>CWBM</th>
<th>MBM</th>
<th>CUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>UQI, set A</td>
<td>0.9945</td>
<td>0.9943</td>
<td>0.9876</td>
<td>0.9938</td>
<td>0.9915</td>
<td>0.9925</td>
</tr>
<tr>
<td>UQI, set B</td>
<td>0.9895</td>
<td>0.9892</td>
<td>0.9848</td>
<td>0.9889</td>
<td>0.9837</td>
<td>0.9843</td>
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<tr>
<td>UQI, set C</td>
<td>0.9974</td>
<td>0.9972</td>
<td>0.9991</td>
<td>0.9970</td>
<td>0.9961</td>
<td>0.9964</td>
</tr>
<tr>
<td>UQI, set D</td>
<td>0.9965</td>
<td>0.9963</td>
<td>0.9963</td>
<td>0.9958</td>
<td>0.9949</td>
<td>0.9953</td>
</tr>
<tr>
<td>UQI, set E</td>
<td>0.9922</td>
<td>0.9919</td>
<td>0.9433</td>
<td>0.9911</td>
<td>0.9885</td>
<td>0.9891</td>
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<tr>
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<td>3rd</td>
<td>5th</td>
<td>4th</td>
</tr>
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</table>

DOFE always the best, DFF always the worst
Some of the experiments performed

SYNTHETIC DATA SETS WITH GROUND TRUTH

Brodatz textures

METRIC AUQI

<table>
<thead>
<tr>
<th>AUQI, SET</th>
<th>METRIC</th>
<th>SET</th>
<th>DOFE</th>
<th>SF</th>
<th>DFF</th>
<th>CWBM</th>
<th>MBM</th>
<th>CUR</th>
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</thead>
<tbody>
<tr>
<td>AUQI, set A</td>
<td>AUQI</td>
<td>0.8822</td>
<td>0.8823</td>
<td>//</td>
<td>0.8831</td>
<td>0.8917</td>
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<tr>
<td>AUQI, set B</td>
<td>AUQI</td>
<td>0.6538</td>
<td>0.6541</td>
<td>//</td>
<td>0.6543</td>
<td>0.6688</td>
<td>0.6674</td>
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</tr>
<tr>
<td>AUQI, set C</td>
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<td>0.9220</td>
<td>0.9223</td>
<td>//</td>
<td>0.9230</td>
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<td>0.9270</td>
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<tr>
<td>AUQI, set D</td>
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<td>0.9306</td>
<td>0.9308</td>
<td>//</td>
<td>0.9315</td>
<td>0.9360</td>
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<tr>
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<td>6th</td>
<td>3rd</td>
<td>5th</td>
<td>4th</td>
<td></td>
</tr>
</tbody>
</table>

DOFE always the best, DFF always the worst

Some of the experiments performed

REAL WORLD DATA SETS (GROUND TRUTH IS NOT AVAILABLE)

FLY EYE  INTESTINE  PANCREAS  LASER  SPHEROID

METRIC AUQI

<table>
<thead>
<tr>
<th>METRIC, SET</th>
<th>METRIC</th>
<th>SET</th>
<th>DOFE</th>
<th>SF</th>
<th>DFF</th>
<th>CWBM</th>
<th>MBM</th>
<th>CUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUQI, set “fly eye”</td>
<td>AUQI</td>
<td>0.9021</td>
<td>0.8996</td>
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<td>0.9062</td>
<td>0.9086</td>
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</tr>
<tr>
<td>AUQI, set “intestine”</td>
<td>AUQI</td>
<td>0.8286</td>
<td>0.8278</td>
<td>//</td>
<td>0.8542</td>
<td>0.8466</td>
<td>0.8393</td>
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</tr>
<tr>
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<td>AUQI</td>
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<td>0.9413</td>
<td>//</td>
<td>0.9557</td>
<td>0.9561</td>
<td>0.9495</td>
<td></td>
</tr>
<tr>
<td>AUQI, set “laser”</td>
<td>AUQI</td>
<td>0.9278</td>
<td>0.9271</td>
<td>//</td>
<td>0.9306</td>
<td>0.9422</td>
<td>0.9413</td>
<td></td>
</tr>
<tr>
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<td>AUQI</td>
<td>0.8557</td>
<td>0.8524</td>
<td>//</td>
<td>0.8947</td>
<td>0.8692</td>
<td>0.8904</td>
<td></td>
</tr>
</tbody>
</table>
Some of the experiments performed

REAL WORLD DATA SETS (GROUND TRUTH IS NOT AVAILABLE)

<table>
<thead>
<tr>
<th>METRIC, SET</th>
<th>DOFE</th>
<th>SF</th>
<th>DFF</th>
<th>CWBM</th>
<th>MBM</th>
<th>CUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUQI, set “fly eye”</td>
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<td>6th</td>
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<td>5th</td>
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<td>AUQI, set “intestine”</td>
<td>2nd</td>
<td>1st</td>
<td>6th</td>
<td>5th</td>
<td>4th</td>
<td>3rd</td>
</tr>
<tr>
<td>AUQI, set “pancreas”</td>
<td>1st</td>
<td>2nd</td>
<td>6th</td>
<td>4th</td>
<td>5th</td>
<td>3rd</td>
</tr>
<tr>
<td>AUQI, set “laser”</td>
<td>2nd</td>
<td>1st</td>
<td>6th</td>
<td>3rd</td>
<td>5th</td>
<td>4th</td>
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<td>1st</td>
<td>6th</td>
<td>5th</td>
<td>3rd</td>
<td>4th</td>
</tr>
</tbody>
</table>

Depth-of-focus

CONCLUSIONS

- We validated an approach for comparing different methods also using real sets of images (not correlated of ground truth)
- We proposed a new method for extending the depth of focus of the microscope achieving better, or at least comparable, results with the state-of-the-art methods

PUBLICATIONS


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Courses, summer schools

- **Short course of Robotics**  
  C. Melchiorri et al., 4 credits
- **Devices, Circuits and Systems for Energy Efficiency**  
  C. Fiegna, M. Tartagni, A. Costanzo, M. Benini, 2 credits
- **Reconfigurable Processors**  
  F. Campi, C. Mucci, 1 credit
- **Neural Networks: Theory and Applications**  
  M. Ursino et al., 3 credits
- **Advanced metrology: metrological proof and measure uncertainty**  
  M. Saddemi et al., 2 credits
- **ICVSS 2010 Computer Vision Summer School**  
  Scicli (Ragusa), Italy, July 12-17, 2010, 2 credits
- **CIMST 2010 Interdisciplinary Summer School on Bio-medical Imaging**  
  ETH Zurich, Switzerland, September 6-17, 2010, 5 credits

Total 19 credits
Stay abroad, conferences

LMSC, ETH Zurich, Switzerland
May 9 – August 26, 2011 and May 7 – August 8, 2012

EMBS 2011, August 30 - September 3, Boston, Massachusetts, USA
33rd IEEE Engineering in Medicine and Biology Society

CIBCB 2011, April 11-15, Paris, France
8th IEEE Computational Intelligence in Bioinformatics and Computational Biology

BSI 2012, July 2-4, Como, Italy
7th International Workshop on Biosignal Interpretation

Journals


Conference Proceedings

F. Piccinini, A. Bevilacqua, K. Smith, P. Horvath, "Vignetting and photo-bleaching correction in automated fluorescence microscopy from an array of overlapping images", 10th Int. Symposium on Biomedical Imaging (ISBI), San Francisco, USA, April 7-11, 2013.

A. Bevilacqua, F. Piccinini, “Is an empty field the best reference to correct vignetting in microscopy?”, 7th Int. Workshop on Biosignal Interpretation (BSI), Como, Italy, July 2-4, 2012, pp. 267-270.


Next works


Co-tutor in supporting teaching activities

MASTER THESES IN BIOMEDICAL ENGINEERING

- Luigi Caiffa, March 2013, “Studio di classi di sferoidi multicellulari di carcinoma polmonare epidermoide in radiobiologia”
- Davide Pollini, March 2012, “Ricostruzione di immagini di broncosfere in microscopia ottica con tecniche di estensione della profondità di fuoco”
- Marco Marchetti, March 2012, “Segmentazione automatica di regioni in immagini istologiche”
- Andrea Giorni, March 2012, “Misure di segnali fluorescenti per l’analisi in microscopia dell’espressione genica in biologia sintetica”

BACHELOR THESES IN BIOMEDICAL ENGINEERING

- Alessandro Cedioli, March 2012, “Segmentazione automatica di regioni in immagini istologiche”
- Ilaria Fantigrossi, October 2012, “Analisi temporale di caratteristiche morfometriche estratte da immagini di broncosfere sottoposte a differenti trattamenti radiobiologici”

MASTER THESIS IN COMPUTER SCIENCE ENGINEERING

- Carlo Busa, December 2011, “Automatic detection of cancerous regions in histopathological images”

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THANK YOU!

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THANK YOU!