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## Developmental cell lineage dynamics in Bicuspid Aortic Valve disease

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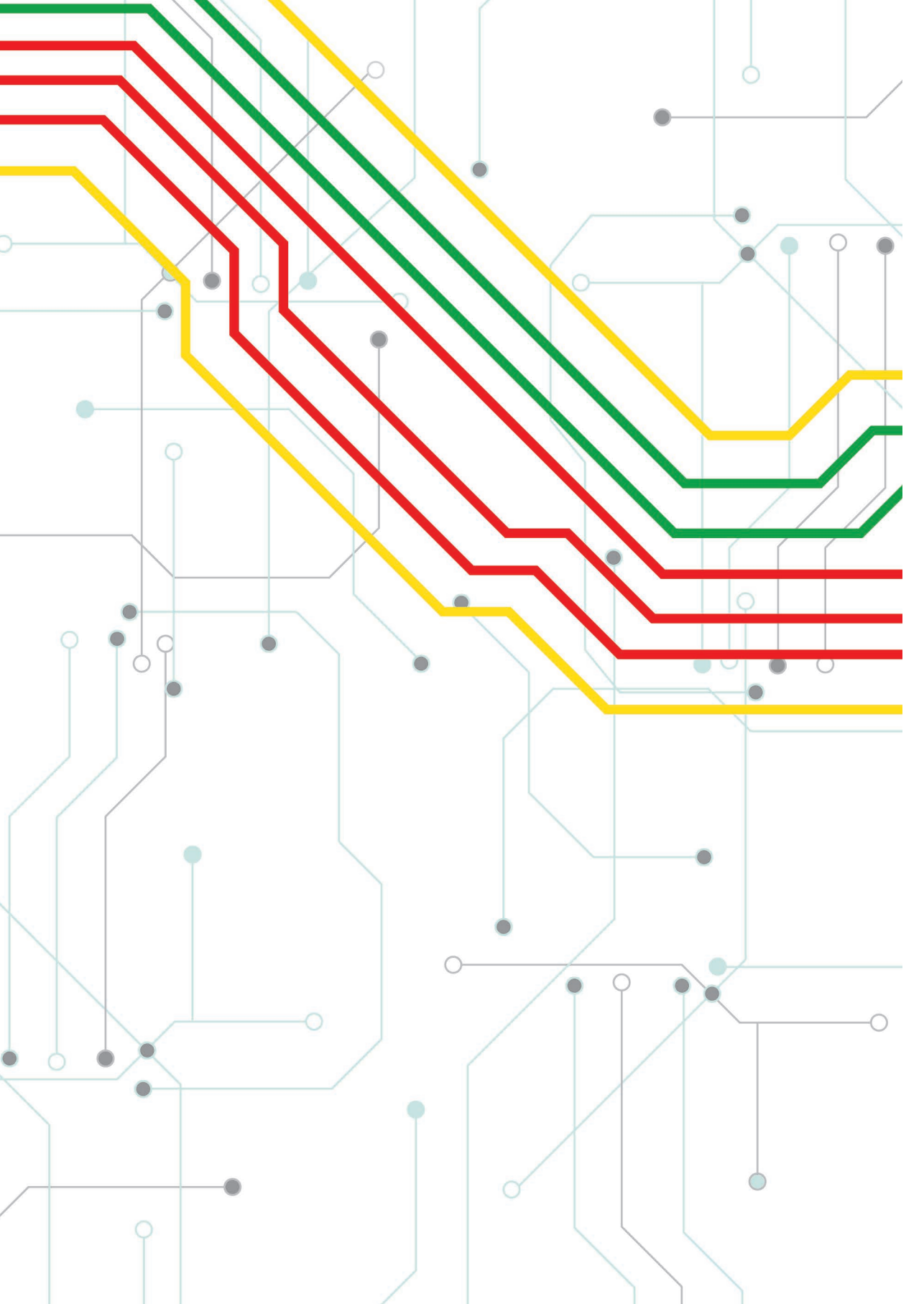
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# Chapter 3

## Fluorescent Nuclei Measurements Macro (FNMM), a tool for automated cell quantification in ImageJ

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**Software Impacts**

**Software:** <https://github.com/J-PTRson/Cell-Image-Analysis/tree/master/FNMM>

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## Abstract

A common requirement, in field of life sciences, is the quantification of specific cells in a given fluorescent microscopy image. ImageJ is a popular tool for researchers to develop custom scripts for automated cell quantification. Validating the quality of cell quantification measurements is a common challenge for such researchers. The Fluorescent Nuclei Measurements Macro (FNMM) was developed to assist researchers performing cell quantification by presenting the results in an intuitive manner whilst simultaneously facilitating processed data collection and measurement assurance.

# 3

## 3.1 Introduction

Since the introduction of digital microscopy the methods for image analysis have been evolving continuously. Advanced microscopes can now generate large amounts of multi-channel fluorescence images of cells. Although analytical methods have changed over time, most questions in cell biology are still based on accurate cell quantification. Software tools like ImageJ (*Rueden et al., 2017*) allow researchers to quickly develop custom methods for automated image analysis. Even though the flexibility of ImageJ allows for highly sophisticated method development, researchers new to software development may struggle to incorporate the collection of data to review such scripts. Around 90% of scientists developing software are self-taught (*Wilson et al., 2014*) and as a result often fall short to ensure the standards required for reproducibility and reusability of technical analysis (*Jiménez et al., 2017*). Whilst methodological instruments and laboratory equipment is often validated, ensuring technical accuracy and reliability of developed software can be challenging (*Roberts, 1994*). There are many online tutorials available to aid researchers in performing automated cell counting using ImageJ, yet these often fail to introduce the aspects of measurements assurance to validate analytical performance. A key conclusion from the cell counting workshop by the National Institute of Standards and Technology and US Food And Drug Administration recommends users to consider the purpose of their cell counting measurement and design the most appropriate method, including measurement assurance strategies as there is currently no single approach for obtaining high quality cell count measurements (*Lin-Gibson, Sarkar and Elliott, 2018*).

For this reason, we have developed a software script specific to ImageJ to facilitate collection of process-data for measurement assurance and simultaneously aid researchers in exercising

good research practices. This script was designed with a very relatable research question in mind. That is, given any fluorescent microscopic image, how many green fluorescent protein (GFP) positive cells are there relative to all cells in that image. In our situation we have used this macro to examine fluorescent cell lineage distributions throughout embryonic development (Peterson *et al.*, 2018). However, this macro has been built to allow for easy adaptation for use in any situation which addresses questions regarding multi-channel fluorescent cell populations.

## 3.2 Description

The Fluorescent Nuclei Measurements Macro (FNMM) was designed to estimate a measure of GFP positive cells within a given cell population using Fiji (Schindelin *et al.*, 2012) (ImageJ). This is accomplished by analysing the area of 4',6-diamidino-2-phenylindole (DAPI) positive nuclei enveloped by cytoplasmic GFP versus the total area of all DAPI positive nuclei within a given region of interest (ROI).

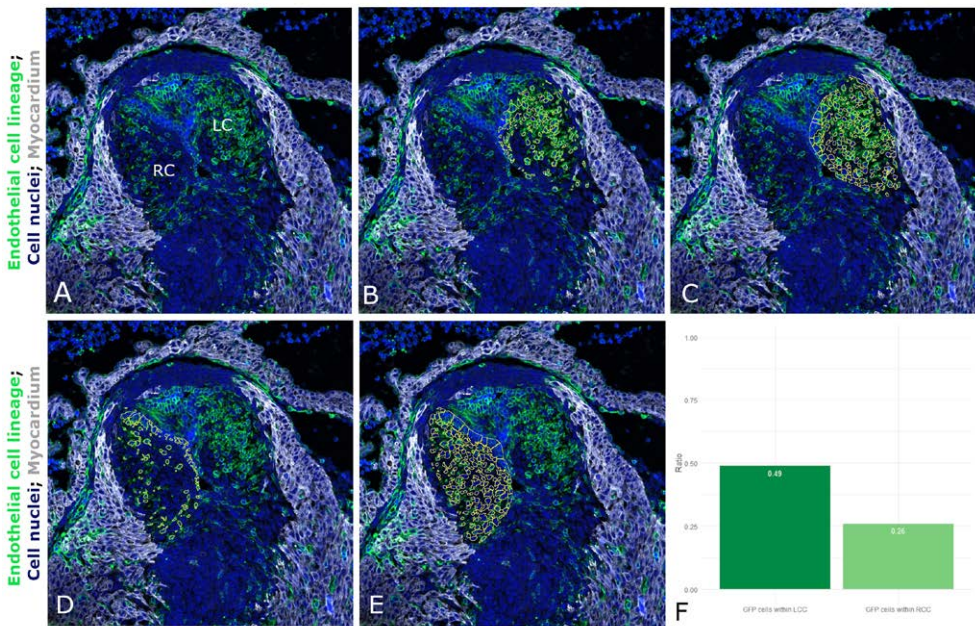
The macro facilitates and standardizes image analysis of fluorescent images by allowing users to apply regional masks to define a specific region of interest to perform the analysis. To achieve cell type analysis, this macro analyses multichannel composite images and depends on the presence of a general nuclear cell staining, like the commonly used DAPI staining, embedded in a dedicated image channel, in combination with a cytoplasmic cell staining (like GFP) in a secondary image channel. A user defined threshold is used to identify positive cells in the DAPI channel which creates a mask of all positive cell nuclei. A similar process is performed to determine the areas of GFP positive cytoplasm in the GFP channel. By combining the results of both the DAPI and GFP masks an image filter is constructed to identify the DAPI positive nuclei within GFP positive cell bodies. Using this filtered image, area measurements are performed to calculate the nuclear area of all GFP positive cells. This result is offset to the nuclear area of all DAPI positive cells to derive a ratio of GFP positive cells within the selected ROI.

All results are recorded in .CSV format and visual representations of the labelled area measurements are exported to a local folder for measurement assurance and peer inspection (Fig. 3.1). This approach ensures data collection of original input images, as well as the labelled output images to allow for more transparent cell image quantification.

### 3.3 Impact

The quantification of certain cell types using fluorescent imaging is a common explorative procedure during biomedical research. Current microscopes can collect a large volume of high resolution images of cells in a single day. Whether it is for optimizing transfection efficiencies in vitro, ex vivo morphologic analysis, in vivo expression analysis or high throughput compound analysis the need to label and quantify cells persist (**Fig. 3.1, Fig. 3.2**). Nevertheless, there remain bottlenecks at the image analysis stage. Automated cell image quantification presents a solution to increase analytical throughput. Yet accurate validation of automated quantification results is still a challenge. This script allows researchers looking to perform cell quantifications to address the measurement uncertainty related to automated image

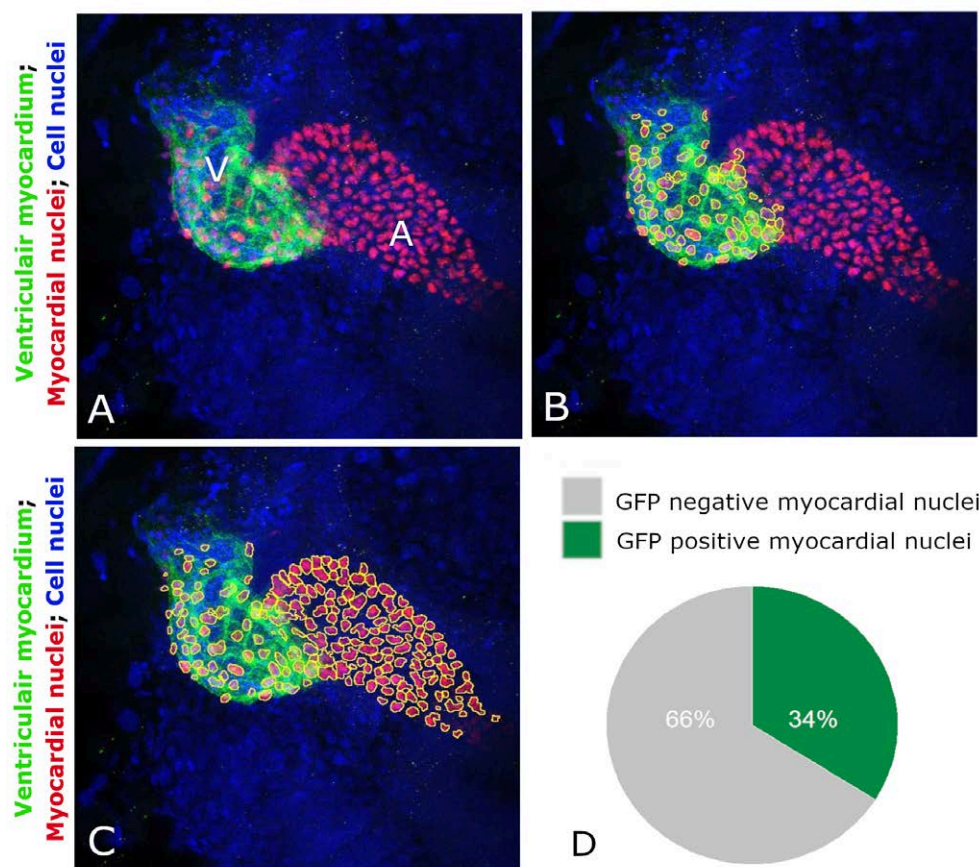
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**Figure 3.1** Using FNMM to examine cell lineage distribution within the aortic valve.

**A:** The original input image is a transversal section of the aortic valve in an embryonic day 13.5 *bl6.Tie2Cre;mTmG* mouse embryo showing the right coronary cusp (RC) as well as the left coronary cusp (LC). **B:** The LC was selected for analysis, here the cell nuclei (blue) of endothelial derived cells (green) have been quantified and outlined in yellow for visual examination. **C:** Here, all nuclei within the LC were quantified based on the cell nuclei staining. **D-E:** Similar output files as B and C, however, analysis was performed on the RC. **F:** Comparing the ratio of green to blue cells allows for rapid analysis of relative cell distributions in morphological structures.





**Figure 3.2 Using FNMM to quantify myocardial cell contribution to the atrium and ventricle during development.** **A:** Original input image of a 48 hours post fertilization zebrafish heart showing the ventricle (V) and atrium (A). **B:** Myocardial nuclei (red) within the ventricle (green) were quantified and outlined in yellow. **C:** Here, the total area of myocardial cells is measured. **D:** Following this approach, it can be determined that 34% of myocardial cells in the heart contribute to the ventricle whilst 66% of cells contribute to the atrium at this developmental stage.

analysis. This script presents the analytical results in an intuitive manner whilst simultaneously facilitating processed data collection and measurement assurance. This allows users to more easily evaluate the effects of variation in input parameters on the accuracy and reliability of the intended cellular measurements. Knowing that the analysis performed according to expectation improves the pursuit of the existing research questions as concerns regarding measurement error is minimized and can be retrospectively reviewed by multiple peers if required. Moreover, the general-purpose design of this script allows researchers to easily pursue new research questions through reuse of public datasets derived from other studies, like those published

at the Image Data Resource (IDR) (<https://idr.openmicroscopy.org/>). Studies examining in-vivo cell dynamics such as the work of Duim et al. (2015) or Ge et al. (2020) could benefit from cell quantification scripts like ours as the heterogeneous morphology of cardiac tissue following myocardial infarction requires dynamic selection of ROIs due to local tissue adaptation. The dynamic application of this script and easy customization options provided through ImageJ may be more advantageous than customization options provided by other Image analysis tools such as CellProfiler (Carpenter et al., 2006). A comparison of 15 free image analysis tools published by Wiesmann et al. describes that tools such as ImageJ include more functionality but lack more usability due to less user guidance compared to the CellProfiler (Wiesmann et al., 2015). Our script addresses this user guidance through an interactive workflow with optional dialog requests for researchers less comfortable with automated cell quantification. An early version of this script has already been successfully used to enable the study of developmental changes in cell lineage distribution in *Nos3<sup>-/-</sup>* mice (Peterson et al., 2018). In our ongoing research we are deploying this script for cell analysis of congenital heart disease in mice.

### 3.4 Limitations and potential improvements

The main limitation is that this script currently only supports 2D image analysis. Nevertheless, the basic premise on which this script was built allows for rapid customization by its users. Another potential limitation is that this script was designed solely for fluorescence cell analysis. Histological staining such as hematoxylin eosin (HE) are incompatible using this method. As such, one of our goals is to continue development for efficient analysis of histological images in the future.



<b>Code metadata description</b>	
<b>Current Code version</b>	V2.0.0
<b>Permanent link to code / repository used of this code version</b>	<a href="https://github.com/SoftwareImpacts/SIMPAC">https://github.com/SoftwareImpacts/SIMPAC</a>
<b>Legal Code License</b>	MIT License
<b>Code Versioning system used</b>	Git
<b>Software Code Language used</b>	IJM
<b>Compilation requirements, Operating environments &amp; dependencies</b>	Fiji (ImageJ v1.52p)
<b>If available Link to developer documentation / manual</b>	<a href="https://github.com/J-PTRson/Cell-Image-Analysis/tree/master/FNMM">https://github.com/J-PTRson/Cell-Image-Analysis/tree/master/FNMM</a>
<b>Support email for questions</b>	<a href="mailto:j.c.peterson@lumc.nl">j.c.peterson@lumc.nl</a>