DEVELOPMENT OF A HYPERSPECTRAL OPTICAL SYSTEM FOR FLOW CYTOMETRY
Flow cytometry is a powerful method of biomedical imaging. It is being actively utilized in immunophenotyping, cell sorting, and cancer diagnostics among other applications. Flow cytometry market is rapidly developing and new approaches to signal collection are invented and taken into utilization. Among the latest trends is introduction of imaging into flow cytometrical devices to increase the information content of the studied sample.

However, implementation of an additional imaging unit in addition to existing optical subsystem increases the bulk of the instrument. A possible approach not only to decrease the size of the device but also to increase the information content of the recorded samples is the implementation of the hyperspectral imaging to flow cytometry.

In the course of this work, an optical setup for evaluating particles imaging with hyperspectral camera is established. A basic fluidics setup, not featuring hydrodynamic focusing, is also configured for testing purposes. Particles flowing through the flow cell are imaged with alternative cameras to compare on the quality and information content of the recorded data.

Imaging is performed with red-green-blue and hyperspectral cameras. The setup is configured in a way to allow acquisition of images with both imaging methods simultaneously for more direct comparison. In addition to mentioned methods, the studied sample is imaged with multimode plate reader to offer a comprehensive comparison of self-build imaging solutions to imaging method available in the market.

The results reveal the possibility to localise imaged particles and differentiate those from the surrounding fluid based on the spectra recorded with hyperspectral camera. It has also been demonstrated achievable to differentiate between different intensity levels of recorded particles for hyperspectral recordings, a capability not exhibited by alternative imaging methods in this research.

The peak of the imaged fluorophore emission, however, has either been shifted in the recorded images or not identifiable due to having lower intensity than the excitation laser light. Additional investigation is required to confirm the nature of the recoded spectrum shift.

Based on the obtained results, further research could focus on investigating techniques to distinguish the fluorophore signal from the background noise. Those could possibly involve filtering implementation coupled with refinement of the flow cell configuration. Potentially, multiple sample cells could be imaged simultaneously with this method, which would increase its flow-through.

Keywords: imaging flow cytometry, biomedical imaging, hyperspectral imaging

The originality of this thesis has been checked using the Turnitin OriginalityCheck service.
PREFACE

This thesis was carried out in collaboration with Modulight Corporation to be included as a part of Master's Degree in Biomedical Engineering in Tampere University. The research was done in October 2022 – May 2023.

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Finally, as “behind every great man there is a great woman”, behind me there is my family. Nothing would have been possible without your support and encouragement. Lastly, thanks to Andrey for being my “great man”.

Tampere, 7 May 2023

Anna Filipchuk
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## LIST OF SYMBOLS AND ABBREVIATIONS

### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ADC</td>
<td>Analog-to-Digital Converter</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid Leukaemia</td>
</tr>
<tr>
<td>APD</td>
<td>Avalanche Photodiode</td>
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<tr>
<td>CCD</td>
<td>Charge-Coupled Device</td>
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<tr>
<td>CTC</td>
<td>Circulating Tumour Cell</td>
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<tr>
<td>DR</td>
<td>Dynamic Range</td>
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<td>FC</td>
<td>Flow Cytometry</td>
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<td>FWHM</td>
<td>Full Width Half Maximum</td>
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<td>HS</td>
<td>Hyperspectral</td>
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<tr>
<td>IFC</td>
<td>Imaging Flow Cytometry</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
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<tr>
<td>LB</td>
<td>Liquid Biopsy</td>
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<tr>
<td>LED</td>
<td>Light Emitting Diode</td>
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<tr>
<td>NPM</td>
<td>Nucleophosmin</td>
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<tr>
<td>PMT</td>
<td>Photomultiplier Tube</td>
</tr>
<tr>
<td>RGB</td>
<td>Red-Green-Blue</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal-to-Noise Ratio</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scatter</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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### Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$A$</td>
<td>Cross-sectional area of fluid perpendicular to the flow</td>
</tr>
<tr>
<td>$i_{\text{max}}$</td>
<td>The largest nonsaturating signal in an image</td>
</tr>
<tr>
<td>$i_{\text{min}}$</td>
<td>The lowest nonsaturating signal in an image</td>
</tr>
<tr>
<td>$\mu_{\text{sig}}$</td>
<td>Average signal value</td>
</tr>
<tr>
<td>$Q$</td>
<td>Volumetric flow rate</td>
</tr>
<tr>
<td>$\sigma_{\text{sig}}$</td>
<td>Standard deviation of the signal</td>
</tr>
<tr>
<td>$v$</td>
<td>Average velocity</td>
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1. INTRODUCTION

The advancement of modern technology has greatly enhanced our ability to gather and analyse vast amounts of data. In the medical field, this data is crucial for accurate diagnostics and effective treatment planning. In biological research, it allows us to uncover previously unknown aspects of living organisms. While there are many alternative biological data acquisition methods developed and utilized – further technological advancements make it possible to improve the existing techniques even further. An example of such improvement is implementation of hyper- or multispectral imaging to the fields of biomedical imaging where this technology has not yet been applied. In particular, to the flow cytometry analysis.

The flow cytometry market has experienced significant growth [1], largely driven by the active research trend among companies seeking to establish themselves as market leaders. This can only be accomplished if the flow cytometrical supplier would offer unique device from the technological point of view. The competition for such uniqueness has led to focus on expanding the number of lasers and detectors employed in cytometers, which is limited by the availability of fluorescent markers. Considering these limitations, the latest trends in this field have been shifted to focus on increasing the information content of collected data while simplifying device complexity [2]. Hyperspectral imaging represents an attractive technology for achieving these goals.

Hyperspectral imaging provides an opportunity to collect spectral information across a two-dimensional image. The obtained spectra present us with valuable data not visible to human eye. Since first introduction of the term “hyperspectral imaging” in mid-1980s [3], the underlying technology has been actively developed. Recent advances suggest further increase in application fields and high demand for this technology in the future.

Despite the increase in the application area of hyperspectral imaging, there has been little prior research conducted on the evaluation of its applicability to flow cytometry [4], [5]. This thesis seeks to address this missing gap by examining the applicability of hyperspectral imaging to flow cytometry in a comprehensive manner.

Within this thesis, a constructed imaging system will be utilized to investigate hyperspectral imaging in flow cytometrical context. Both fluorescent and non-fluorescent particles will be captured using red-green-blue and hyperspectral cameras. The acquired images will be compared in terms of their information content. Specifically, the potential to determine the composition of fluorescence markers in the examined sample will be
assessed by analysing the recorded point spectrum obtained with the hyperspectral camera. Accomplishing this analysis will fulfil the primary objective of this thesis, which is to ascertain whether hyperspectral imaging provides a comprehensive approach for data analysis in flow cytometry.
2. THEORETICAL BACKGROUND

Biological imaging techniques enable acquisition of structural, functional, and dynamical properties of biological systems [6]. Examples of those could be visualization of cells movement and protein composition. The information acquired via biological imaging is vital in understanding work mechanisms in living systems, disease diagnostics and treatment.

There are various biological imaging techniques, such as: light and fluorescent microscopy, electron microscopy, x-ray crystallography, micro-computed tomography, x-ray fluorescence imaging and flow cytometry. In the course of this work, we are going to focus on the latter. However, it should be noted that with certain adjustments the optical system at hand can be applied to other imaging modalities as well.

2.1. Flow cytometry

Flow cytometry (FC) is an established technique used in quantitative and qualitative analysis of cells. By the means of it – data on cell’s size, morphology and composition can be deduced. Many scientists have contributed to the development of what is currently known as flow cytometry. While it is hard to identify a single person responsible for the invention, the first one to work on fluorescent-based cytometry was Wolfgang Göhde in 1968 [7]. Since then, flow cytometry has been gaining worldwide recognition and is now acknowledged as a powerful technique vastly utilized in immunology, virology, cancer and molecular biology, and disease monitoring [8].

The technologies behind the technique are continuously improving to keep up with the latest technological breakthroughs and simultaneously increasing the application areas of flow cytometry even more. In this section we are going to review the basic working principle of flow cytometers along with the latest technological advances utilized in the field.

2.1.1. Operation principle

To perform analysis on the biological cells of interest, they should be first diluted with pH buffer. The obtained solution is then pumped through a narrow cell chamber, referred to as flow cell [9]. Flow cells come in various shapes and are mostly made of quartz or glass. Their key purpose is constraining and supporting the flow of particles.
The imaging procedure in flow cytometry can be then described as following: laser light shines on the flow cell, through which cells/particles of the studied matter pass through, as seen in Figure 1. Part of the original light which passes through is diffracted depending on the properties of the particle it interacts with. The intensity of light right after the flow cell and to the side from the flow cell is measured with forward scatter detector (FSC) and side scatter detector (SSC).

![Figure 1. Basic principle of flow cytometry.](image)

Based on intensity values recorded by FSC and SSC detectors - such properties as size and shape complexity of the studied cell can be analysed (Figure 2). While there is more than one factor influencing the determination of cell physical properties – it is conventionally assumed that the amount of light recorded by FSC detector is approximately inversely proportional to cell's size (Fig. 2 (a)). In the meanwhile, the amount of light recorded by SSC is approximately proportional to the cell's granularity and complexity (Fig. 2 (b)) [9].
Apart from determination of physical properties, flow cytometry enables gaining understanding of the cell's chemical composition. For that, fluorescence measurements are performed. Very low number of chemical components of interest are fluorescent on their own. Thus, to perform fluorescence analysis, samples must be additionally prepared for fluorescent measurements by various techniques during which a fluorescent component is added to the cell for labelling purposes. Labelling can be done by various means, among which are: staining with fluorescent dyes, staining with fluorescently conjugated antibodies, transfection and expression of fluorescent proteins and quantum dot probes [8].

Fluorophores or fluorochromes are photoreactive chemicals, which produce photon emission when excited with a specific amount of energy [10]. In flow cytometry narrow-bandwidth laser light is typically used for excitation, which leads to stable and predictable fluorescent light emission indicative of the presence of a certain chemical component within a studied specimen. Conventionally, separate detectors are then employed to detect the occurrence of each present fluorophore emission wavelength. The detectors are arranged in a similar manner as FCS and SSC detectors (Figure 3) and provide data on the level of intensity of each present wavelength i.e., level of fluorophore expression in the sample [9].

**Figure 2.** a) **Forward scatter**, b) **Side scatter**.
Figure 3. Detectors arrangement in flow cytometer.

In conventionally used flow cytometers data is presented as two parameter histograms or dot plots, where intensity values from different detectors are plotted against each other (Figure 4). Each dot on the plot represents a cell and location of the dot indicates how strongly the cell exhibits certain fluorophore or how much light was scattered from it in FSC/SSC. If the parameters of the experiment are selected correctly, we would likely see regions of dots grouped together. Those indicate clusters of the same types of cells. The more cells are analysed – the more evident the groups become. It is then possible to select a region of cells within one plot and apply it to other parameters within the experiment for more representable analysis. This technique in data analysis is referred to as gating [8]. Gating provides usefulness when a group of cells clearly exhibits the presence of a certain fluorophore, and we would want to analyse the results for this particular group for other parameters in the experimental run.

Figure 4. a) Mouse dendritic cells, migratory and resident on the lymph node, no gating b) Mouse B and T cells, with gating. (Courtesy of Laura Kummola, Tampere University)
An example of flow cytometrical data without gating representing mouse dendritic cells, migratory and resident on the lymph node, is presented in Figure 4 (a). Gating is applied in Figure 4 (b) to separate mouse B and T cells, as a result, two separate cell groups can be clearly differentiated.

While this kind of data representation is convenient and easy to read for low number of parameters, analysis gets more laborious and time-consuming as the number of parameters increases. In addition, since intensity values are only plotted against each other, the data would be relative in nature, rather than absolute. This drawback can be avoided in imaging flow cytometers discussed further in Section 1.1.3.

2.1.2. Conventional flow cytometry

Any flow cytometer consists of three main subsystems: electronical, fluidical, and optical. Whereas we would focus on the optical subsystem in this work, let us review the purpose of each to better understand the internal operation mechanisms.

Electronics is responsible for conversion of detector signals. Electronics subsystem consists of pre-amplifier, amplifier, analogue-to-digital converter (ADC) and data processor [11]. Photons from scattering/fluorescence emission are converted to electrons on the detector. After this conversion photocurrent leaves the detector and travels to the amplifier to be further converted to a voltage pulse. The pulse undergoes analogue-to-digital conversion after which a digital number reaches the computer and can be subsequently analysed [12]. This procedure is well-established and is consistent across various flow cytometers.

Fluidics establishes the flow of particles, enabling analysis of thousands of cells per second [13], which gives cytometers a huge speed advantage over bulk analysis approaches. Basic fluidics arrangement includes components such as flow pump, focusing device, flow cell, tubing connections, and waste management arrangement. Subsystem operation starts at sample delivery. The sample stream is then positioned correctly to ensure measurements of one particle at a time. Positioning is conventionally done via hydrodynamic focusing, where the core sample stream is surrounded by a fast-moving sheath fluid stream (Figure 5). Following the properties of fluid dynamics, the fluids do not mix but instead form a two-layer stable flow [14]. Apart from fast delivery the approach also eliminates capillary clogging.
Although hydrodynamic focusing is still the most abundantly used focusing method, parallel control of two flows (sample and sheath) is cumbersome. In addition, utilization of sheath fluid requires its regular replacement and disposal. There are ongoing research works on sheath fluid free cytometry development, which would decrease overall system size and make the operation more intuitive. Among the most promising alternatives developed are acoustic, dielectrophoretic, inertial, and magnetic focusing approaches [15], [16].

The last and the most crucial subsystem of flow cytometer is optics. It is often referred to as the heart of the flow cytometer. It can itself be further divided into three main constituting parts: illumination, fine-tune optics, and detection units.

Historically, broad-band light sources, such as arc lamps and halogen lamps, have been used for illumination [17]. However, due to excessive light noise and lowered sensitivity – preference was given to narrow-band excitation sources. Water-cooled Argon laser was one of the first lasers employed for cytometrical applications [18]. Being proven effective, multiple wavelength fluorescent excitation was gaining more and more attention. Current cytometers typically employ at least four lasers, while bigger systems can include seven or more. Laser quality plays a big role, since narrower beam would lead to more predictable interaction with fluorophore and require less filtering.

Fine-tune optics refers to the setup of optical filters, lenses, and mirrors. Its choice would fully depend on the used illumination and detection systems, number of wavelengths utilized and geometrical constraints of the developed platform. Due to an ongoing simplification trend – it would be preferable to decrease the amount of additional filtering components to achieve both: simplify system’s tuning and keep the measured data intact for possible additional data extraction.
Detection system determines how much information will be recorded and how accurate this information will be. The most popular detector choice is photomultiplier tubes (PMTs) due to their low-noise and good sensitivity characteristics [19]. Avalanche photodiodes (APDs) have been also gaining wider attention due to reported higher sensitivity in comparison to PMTs.

2.1.3. Imaging flow cytometry

Flow cytometrical structure described in Section 1.1.2 can be referred to as conventional. Most flow cytometers provide end-point measurements only and morphological properties are analysed relatively inside the measured population. The lack of morphological details can be addressed with an alternative technique – imaging flow cytometry (IFC).

Basically, IFC combines functionality of conventional flow cytometry and fluorescent microscopy. This means that apart from the presence of a certain fluorophore - its distribution within the cell would be recorded as a 2D image. In addition to fluorescence images some devices have the capability to capture bright field images, providing data with practically the same information content as a bright field microscope would [20].

The detector component in IFC devices is typically a charge-coupled device (CCD) camera [21]. CCD is either present on its own or along with APDs/PMTs. In the cases where fluorescence is recorded along a broad part of the spectrum, several CCD cameras targeting different spectrum regions can be utilized.

IFC has experienced numerous advancements in the past few years. Those include introduction of higher resolution (60x objective), increase in acquisition speed (up to 1000 events/sec), and introduction of additional functionality (switching between FC and IFC) [22]. Those improvements in combination with image acquisition capability account for IFC widespread use, for example, in analysis of nuclear-cytoplasmic translocation, apoptosis quantification, and quantitative analysis of internalized bacteria and protozoan parasites [22]. The range of applications keeps rapidly expanding as technologies behind IFC are being improved further.

IFC’s functionality can be further expanded by introduction of novel hyperspectral imaging technology (HSI) to IFC. The goal of this approach is to increase data information content and simplify the design of optical subsystem. Theory behind hyperspectral imaging is going to be discussed more in the Section 1.2.
2.1.4. **State-of-the-art in flow cytometry**

For more than 60 years since flow cytometers have been first introduced to the market [23], there has been an ongoing competition in diversifying and improving the existing cytometer models. The market rivalry has been provoking the development of the new approaches in flow cytometry and pushing the science frontiers further.

Up to about ten years ago state-of-the-art devices were improved by adding on more lasers and sensors to the module in order to expand the fluorophore detection scope. Among such versatile conventional flow cytometers are, for example, “ID7000” by Sony (featuring seven lasers and up to 186 detectors) [24] and “BD FACSsymphony” by BD Biosciences (featuring nine lasers and up to 50 detectors) [25].

However, at this point the developed conventional flow cytometry devices are approaching their theoretical limit in terms of the number of lasers and detectors it would be feasible to accommodate, since the number of available fluorophore dyes is finite. Instead, the focus has been shifted to introducing additional imaging modalities to the system. As an example of this trend, ThermoFisher Inc. has introduced high-speed brightfield camera to their “Attune CytPix Flow Cytometer”, providing the possibility for simultaneous morphological and compositional data analyses [26]. Brightfield illumination method is commonly used in microscopy, it utilized bright broadband illuminator along with lens system which focuses light onto the specimen. The uniform illumination of the entire field of view is achieved, leading to sharp image production.

Increasing popularity of imaging introduction signalizes of the graduate transition to IFC happening in the FC field. “Amnis ImageStream” product line (Luminex Corporation) can be regarded as a representative example of the state-of-the-art in IFC area [27]. By introducing up to 12 cellular imaging channels – the possibility of in-depth fluorophore distribution analysis has been added to their flow cytometry devices. The realization of imaging is done via spectral decomposition of the beam on CCD cameras. Alternative example of the state-of-the-art level device, released in 2022, is “BD FACSDiscover S8 Cell Sorter” by BD Biosciences [28]. This model utilizes novel “BD CellView Image Technology”, which enables mathematical image reconstruction of waveforms without a need to introduce a camera to the system at all.

Nevertheless, none of the featured devices report the possibility of direct spectrum analysis from the recorded image. Such functionality could offer the potential to further increase the quantity and quality of obtained data. The potential technology, which could offer this feature incorporation is discussed in the following section.
2.2. Hyperspectral imaging

Hyperspectral imaging enables collection of information across the electromagnetic spectrum for each pixel of the recorded image. This indicates that in comparison to standard red-green-blue (RGB) sensors, where data on 3 bands from visible region is collected – the number of bands in hyperspectral cameras extends far beyond that. The idea behind band division is illustrated in Figure 6, which is a simplified representation and in reality, the number of bands goes beyond what is presented. In some cameras the number of bands can go over 1000 [29]. Data in infra-red (IR) and ultra-violet (UV) regions can also be recorded. While an increased number of bands offers an advantage of larger data content, it can also serve as a limiting factor for the speed of HIS. This is due to the fact that for each pixel and each band, enough photons must be gathered to record an image.

Based on collected unique spectral signatures HSI enables highly accurate and relatively fast differentiation between various substances. While the technology has been originally developed for geology and mining, it has now pathed its way to various fields of science and is actively utilized in e.g., quality control in food processing [30].

![Figure 6. Spectral bands of RBG vs. HSI sensors.](image)

HSI can be further categorized based on the underlying acquisition technology for acquiring the three-dimensional dataset of the hyperspectral cube. The key technologies include spectral, spatial, and snapshot scanning [31]. Spectral scanners scan one wavelength after another by exchanging optical filters. This approach enables high resolution images of each spectral band but is relatively time consuming due to the time
required for filter exchange. Spatial scanners obtain the full spectrum of a 1-D line by dispersing light obtained from each line with a dispersive element, which can either be a grating or a prism. The approach allows acquisition of a high number of spectral bands (in the range of 1000s) as well as a high acquisition rate per line. However, to acquire an image of a 2-D object hundreds of frames might have to be taken. Moreover, in case of a moving object – the frame rate of the camera would have to be precisely correlated with the movement speed of the specimen. If this does not hold, blurring, and non-representative data should be expected.

In snapshot HSI the image is captured simultaneously for all spectral bands and there are no scanning or moving parts in this type of camera [32]. Even though the technology was first developed in 1938 [33] it has not gained wide commercial acknowledgement for a long time due to existing technological limitations in spatial resolution. Currently there are cameras on the market offering 24 bands with spatial resolution of 409 x 217 pixels for each band [34]. While these numbers are lower than those observed for spatial and spectral imaging, acquisition during a single integration time offers a huge advantage in terms of method’s applicability for imaging moving objects.

One potential way to implement this type of imaging is a snapshot-mosaic camera. Filters for all present spectral bands are located on top of each camera pixel in a mosaic-like manner (Figure 7). Once the image is captured - a demosaicing process takes place, generating a datacube with separate images for each spectral band present.

![Figure 7. Filter arrangement and demosaicing in a snapshot-mosaic camera.](image-url)
2.2.1. Hyperspectral imaging applications in biological imaging

Introduction of HSI to biological imaging offers an appealing possibility of maximizing data extraction from a single tested sample. Commonly, diagnostics of a certain disease include a series of tests, where each test requires a separate sample taken from the patient. In case of e.g., cancer diagnosis it means that in certain cases – separate biopsy samples would be needed. Not only that extends the diagnostic timeline and delays the start of possibly urgently required treatment – it can also be an exhausting and traumatic experience for a patient due to the nature of tissue biopsy procedure.

One possible implementation field of HSI is liquid biopsy (LB). LB is a vastly researched and promising method for cancer diagnostics. It offers an alternative to traditional biopsies and holds the potential for the detection of cancer through a blood sample only (while blood sampling is most extensively studied – other body fluids can be analysed as well) [35]. Various LB approaches are designed to detect either circulating tumour DNA, exosomes, or whole circulating cancerous cells (CTC). CTCs detection includes fluorophore labelling and analysis of the enriched and stained cells either with sequencing or flow cytometric devices [35], [36]. In both cases additional HSI screening of the samples would offer more precise and detailed data on the specimen characteristics.

In certain cases, localization of the fluorophore inside of the cell can be a vital step for disease diagnostics. One such instance is diagnostic assessment of acute myeloid leukaemia (AML), characterized by translocation of nucleophosmin (NPM) protein from nucleus to cytoplasm (Figure 8). Currently a series of 4 assessment tests is used for AML diagnostics: light microscopy, conventional FL, karyotyping, and fluorescent in situ hybridization. This is essential since conventional flow cytometry on its own would not be able to detect the difference between healthy and affected cells [37]. However, should HSI be applied as the detection method – it would become possible to track NPM precise localization. There is ongoing research featuring implementation of imaging cytometry as the key detector test used in AML diagnostics [37]. Even though this approach is only in the trial phase, the results are very promising, signalizing of the great potential this approach has. Eventually, it should become possible to replace the 4-tests series with IFC analysis only.
2.2.2. Hyperspectral imaging in flow cytometry

As discussed, a combination of spatial and spectral information could be extremely valuable in various diagnostic applications. There is a particular interest in applying HSI to flow cytometry among other biological imaging applications, due to the dynamic nature of FC analysis. Surprisingly, limited research has been done on the subject of HSI usage in flow cytometry. This can likely be attributed to the novelty of employed technology.

The earlier research referencing hyperspectral cytometry [38], [39] focuses on implementation of prisms or diffractive gratings in combination with linear detector arrays. Instead of camera sensors, pluralities of PMTs were employed as detectors, resulting in a limitation in spatial data collection. The hyperspectral cytometry was reported to be built upon the foundations of spectral cytometry, which actively incorporates dispersive components in combination with detector arrays. However, a definitive distinction between spectral and hyperspectral cytometry was not clearly established at that stage.

Following research [40] reports on implementation of a CMOS sensor in combination with line variable filter. A dispersive element was still integrated separately into the setup arrangement, however actual spatial photos of the fluorescent particles were obtained. Linear unmixing was applied for data analysis to distinguish between recorded fluorophores. The HSI method was addressed as promising due to a possibility to differentiate between a larger number of fluorophores with overlapping emission spectra.

From the existing latest research Dittrich and Kraus et el. [4] have got the closest to integrating HSI to IFC. Originally, Kraus et el. [41] has been looking into multispectral imaging of flow cytometrical samples, utilizing an RGB camera as the detector. In their later work [4] a hyperspectral snapshot-mosaic camera was integrated to a self-made flow cytometry optical system assembly. In the reported setup the snapshot-mosaic
camera was connected via optical tube to a 20x objective and then focused on a microfluidic chip. On the opposite side of the chip the excitation light source, namely light emitting diode (LED), on an XY-linear stage was located. The chosen snapshot camera by XIMEA GmbH (Münster, Germany) used Fabry-Pérot interference filters directly applied on the CMOS-sensor. The camera was able to differentiate between 16 channels and, thus, capable to form 16 sub images with one snapshot. Partially contributing to the small size of the camera, the obtained optical system was smaller in size than traditional ones and was able to successfully evaluate 8 different sets of bioparticles. The measured particles lay in the range from 10 to 60 μm. Particles’ clustering was identified as the key imaging obstacle in the conducted research. The work highlighted the lack of a separate dispersive components integrated to the optical system as a big advantage. Such arrangement largely simplified the alignment of the system and overall operation. As mentioned, on the contrary to widely used narrow-bandwidth light sources, LED was utilized as the only light source in the described work. Moreover, the excitation source was located opposite to the detection plane, leading to an inability to assemble the optical module independently of the microfluidic chip.

It would thus be interesting to further research into the development of a hyperspectral optical system for flow cytometry, where laser light would be used for excitation and a hyperspectral camera for detection. Furthermore, it would be feasible to further adjust the geometrical arrangement of the system for its compatibility with various flow cell arrangements. The combination of narrow bandwidth light excitation and hyperspectral camera detection has not been previously reported in literature and is possessing vast application potential.
3. RESEARCH METHODOLOGY AND MATERIALS

Studying potential introduction of hyperspectral imaging to flow cytometry would require assembly of a simplified cytometrical setup. In this section we will investigate required materials and subsystems construction for realization of such assembly. Experimental research methodology will be used throughout the thesis since it enables systematic evaluation of the hyperspectral imaging applicability to flow cytometry.

3.1. Materials

The most crucial parts of flow cytometer discussed in Section 1.1.2 must be present in our setup for the results to be scientifically meaningful. In our experimental arrangement, we will be using materials or parts of the subsystems which are either implemented into flow cytometers on the commercial level or are of sufficient quality to be potentially utilized in them. The block diagram of the system, which we aim to establish can be seen in Figure 9.

![Figure 9. Measurement set-up arrangement. Fluidics components are marked in blue, optical in red, and computer interface in black.](image-url)
3.1.1. Fluidics

First of all, proper fluid flow, in which studied specimen would travel along the imaged area, would need to be established. For the simplification of original methodology testing in this arrangement we would not be utilizing hydrodynamic focusing. An additional motivation for the lack of focusing is an assumption that HS imaging approach would make it possible to image and analyse a region of cells at a time. If this proves to be true and we would, indeed, be able to differentiate multiple cells flowing in the flow at a time, then it could greatly simplify flow cytometer configuration in the future. Primarily, this would align with the trend of reducing the overall size of cytometers, as the focusing subsystem currently occupies a significant portion of the device's bulk.

Our fluidics system would include quartz flow cell as its central component. The utilized flow cell is model 526 by FireflySci Inc. It is a standard flow cell with four polished windows, which can be further modified to meet particular setup requirements. The modifications can be either inlet and outlet shape adjustments or introduction of additional anti-reflective coatings. For our experiment we are going to work with the standard non-modified version for better reproducibility and comparability of the results to existing data. The flow cell dimensions are 4.25 mm x 4.25 mm x 20 mm with the actual fluid channel being 250 x 250 micrometres. The utilization of this specific channel dimension enables comprehensive analysis of the entire spectrum of cells that are typically examined via flow cytometry, ranging in size from 0.2 to 150 micrometres [9]. These dimensions are also considered standard in the industry and are commonly utilized by suppliers to the leading cytometer manufacturers, such as Hamamatsu Photonics and FireflySci Inc.

The selection of the flow cell is driven by industry acceptance, comprehensive analysis range, and standardization. Quartz is reported to have excellent optical properties [42], such as high transparency and low autofluorescence, which makes it an ideal material choice for flow cytometrical research. By working with a standard non-modified cell version, we can ensure better reproducibility and easier comparison of the obtained results to the existing data.

Since there are no commercially available flow cell holders, which exclude hydrodynamic focusing for this type of flow cell, we would need to design one ourselves. The designed holder includes three parts: two side pieces and the baseplate. M3 screws go through all segments creating tension to fasten flow cell in place (Figure 10). The baseplate is 3D printed with black SLA due to its capability to produce exceptionally fine layer thickness, which enables optimal fitting of the flow cell to it. Although the side pieces were originally printed with black SLA as well, the process of cutting threads for the tube fittings resulted
in material crumbling and consequent contamination of the fluid channel. As a result, the material for the side pieces was changed to black PLA.

![Diagram of 3D printed flow cell holder with the flow cell inside.](image)

**Figure 10.** 3D printed flow cell holder with the flow cell inside.

The flow cell holder can be used with the baseplate if the incoming excitation light is positioned in the same plane as the detection component. Alternatively, the baseplate can be removed and the side pieces along with the screws used solely for holding the flow cell in place. This type of assembly is beneficial if the excitation source is located opposite to the detection plane.

Programmable syringe pump NE-1000 by KF Technology is used for driving the sample fluid at a precise flow rate. The pump is capable of producing flow rates commonly used in imaging flow cytometry, ranging from 12.5 microliters/minute to 1000 microliters/minute [43]. In our tests we are going to be mostly using 20 microliters/minute, as it has been empirically identified to be an optimal flow rate enabling image capturing without specimen clumping. Volumetric flow rate \( Q \) can be then found with (1), where \( v \) – average velocity and \( A \) – cross-sectional area of fluid perpendicular to the flow. At 20 microliters/minute for our flow cell parameters \( Q \) equals \( 1.25 \cdot 10^6 \mu m^3/min \).

\[
Q = vA
\]

(1)

Experimental evidence indicates that flow rates above 300 microliters/minute in our assembly can result in fluid leakage. In order to support higher flow rates, a more secure connection between the flow cell and fittings would need to be established. Fluid rates under 300 microliters/minute are, however, sufficient for our purpose.
The chosen syringe pump has a few drawbacks, including limited flow rate range, inconsistent pressure throughout the operation, and limited programmability features. However, the generated flow rates are sufficient for our purposes, especially considering that low flow rates are going to be utilized in our tests. Moreover, the chosen pump was the most cost-effective option among the pressure pumps capable of creating the desired flow rate. It can be noted that at the given stage of evaluation there is no requirement for complex or advanced features incorporated to the pump.

Connections between fluidics subsystem components are established with polytetrafluoroethylene tubing of 0.8 mm inner diameter. The material is a hydrophobic polymer of carbon and fluorine, its inert nature and possession of one of the lowest friction coefficients in solids have made it a popular choice for medical catheters and microfluidics tubing production [44]. The tubing is secured in place via 1/16” flangeless fitting connections. The connection tubes and fitting are of the standardized diameters which are commonly utilized in microfluidics. The studied specimen is diluted in buffer solution and inserted to the system through a syringe mounted on the syringe pump. The obtained fluidics arrangement can be seen in Figure 11.

![Fluidics assembly mimicking crucial flow cytometer fluidic features.](image)

**Figure 11.** Fluidics assembly mimicking crucial flow cytometer fluidic features.

### 3.1.2. Laser and optics

As we have concluded in Chapter 2, narrow-bandwidth excitation with laser source is preferable as it results in better specificity and sensitivity of the measurements. In this arrangement we are going to use 635 nm laser module for the specimen excitation. The
utilized module, ML6500 by Modulight Corporation (Tampere, Finland), produces 635 +/- 3 nm laser light and can reach 1.5 W power. The standard excitation power used in flow cytometry is typically at the level of 50 mW [45], which is also the power level chosen for our experimentation. Apart from supporting optimal power levels, the utilized laser was chosen due to suitable wavelength range, good spectral characteristics and beam profile uniformity.

Light is to be delivered to the flow cell through optical fibre as close positioning of the laser source to the flow cell helps to avoid unwanted beam divergence. To further focus the laser beam on the interrogation region, it was decided to utilise frontal light diffuser. The diffuser helps to scatter and diffuse light in a controlled manner, which is beneficial in our application since it decreases the chances of uneven illumination and hot spots formation.

To focus on the sample stream, it was decided to use an optical microscope, Nikon Eclipse E400. This model is a stable mid-level research microscope, which provides suitable magnification and the possibility to simultaneously image the sample from the camera port and eyepiece, which is advantageous in our application. The device supports the use of various observation techniques, including darkfield, Nomarski DIC, phase contrast, and standard brightfield [46]. In our tests, we are going to be mainly working with the latter. The magnification of the device can range between 10x and 1500x. We are going to be utilizing x10 and x20 magnification, as they enable capturing the full section of fluid channel.

The core imaging component in our setup is Senop Hyperspectral Camera HSC-2. This particular camera model works well in our application due to wide imaging bandwidth and sufficiently high frame rate. The camera images in 500-900 nm wavelength region and can capture up to 1000 spectral bands [47]. The number of imaged wavelength bands at a time along with the wavelength region of interest can be freely selected. This model is frame-based snapshot HS camera, which is also advantageous in our experimental context, due to the possibility to capture a larger sample region at a time. The chosen number of imaged wavelength bands would have a direct effect on the frame rate. The highest image capturing rate is 149 frames/second.

For better comparative analysis RGB camera is utilized alongside with HS. The used camera model is ODC 832 Microscope Camera by KERN Optics. The camera is capable of producing quick and clear colour images. The sensor size of 2592 x 1944 pixels is sufficient to clearly different the imaged particles with the given magnification.
Furthermore, the camera is reported to be well suited for demanding brightfield and fluorescent applications [48], which aligns with our utilization goals for it.

3.1.3. Testing samples
The selection of the testing sample plays a major role in determining which features of the acquired data are to be studied and what results can be expected. As our primary goal includes determination of the applicability of HS camera imaging to the flow cytometry area, we are firstly going to focus on imaging the testing beads instead of the actual living cells. Beads offer a series of advantages when talking about repetitive testing, since they: provide brighter fluorescence, are not prone to photobleaching, and can offer more stable and homogeneous results when imaged.

The testing beads are commonly made of nonporous linear polystyrene, chosen for its optical properties and uniformity [49]. In some cases they are additionally coated with fluorescent dyes. In our experiment we are going to use several types of beads. Those include:

- Nonfluorescent beads of various sizes, ranging from 3.1 to 16.8 micrometres (Spherotech Inc.). This type of beads will be used to check brightfield image of the channel with RGB camera, as well as to analyse if nonfluorescent particles can be distinguished with HS camera.

- Rainbow calibration beads, 8 intensity peaks (Spherotech Inc.), size – 3.1 micrometre. This type of beads is commonly used for flow cytometer calibration. Each particle in the mixture is marked with several fluorophore labels, which enables beads excitation at any wavelength from 365 nm to 650 nm. The particles are marked with the following fluorophores [50]:
  o FITC-A – excited at 488 nm, emitting at 530 nm.
  o PE-A – excited at 561 nm, emitting at 586 nm.
  o PE-TexasRed-A – excited at 561 nm, emitting at 615 nm.
  o PE-Cy5-A – excited at 561 nm, emitting at 670 nm.
  o APC-A – excited at 637 nm, emitting at 670 nm.

Out of these, we would be mainly interested in APC-A as its excitation wavelength aligns well with the chosen laser source.

Moreover, the beads mixture includes particles emitting fluorescence in eight different intensity levels due to being labelled with various concentrations of fluorophores. Hence, it would be possible to examine the potential for discerning different intensity levels using
the HS detector. The distribution of intensities for APC-A as provided by manufacturer can be seen in Figure 12.

![Figure 12. Histogram of APC-A intensity distributions for Rainbow Calibration Particles by Spherotech Inc. [51].](image)

3.2. Experimental design

Utilizing components discussed in Section 1.3, we aim to design an experimental setup and a set of experiments. The objective of experimental series is validation of the hypothesis that application of HS camera in FC can prove to be beneficial and offer advantages over conventional FC. To be able to demonstrate that, we should collect data on the studied specimen with the existing investigation methods and compare it to the data obtained with HS camera. Moreover, it would be interesting to develop more than one setup configurations to be able to justify on how the alteration between those could affect the obtained data.

3.2.1. Proof-of-Concept

HS imaging applicability would be justified if we would have means to fully characterize studied particle’s properties without need for additional assessment with alternative investigation modalities. The chosen specimen has two key characterization areas specified in Figure 13.
Figure 13. Beads’ properties for characterization with proposed methods of investigation.

For our proof-of-concept series of experiments we would perform data collection with commonly used methods of characterization, and then data collection with HS imaging. In both cases we would be characterizing the same specimen, which would allow us to provide a direct comparison on the easiness of usage and quality of obtained data from the appropriate techniques.

First series of tests would be dedicated to characterizing beads with the standard imaging techniques (as stated in Figure 13). We would assess morphological properties of nonfluorescent beads of various sizes with RGB camera. Tests aimed at determination of fluorophore content would be performed with rainbow calibration beads. The fluorophore markers intensity distribution could be taken from the data provided by the beads’ supplier. However, these data would not incorporate the imaging component for the fluorophore markers, which would lead to the need for further evaluation using fluorescent microscopy or similar imaging modality. We will conduct fluorophore imaging using a multimode plate reader “EnSight” by PerkinElmer, which operates by exciting the sample with the desired wavelength and then capturing the sample image with filtered out excitation wavelength.

Second series of tests would be aimed at assessing the HS camera imaging capabilities in the context of FC. To gain a better comprehension of its operational mechanisms and expected output, we intend to first capture some reference images with HS camera in macroscopic and microscopic settings. Then, referring to the proposed setup arrangements (discussed in Section 1.4.2), the fluorescent and nonfluorescent beads flowing through the flow cell would be imaged. Given that the initial hypothesis suggests that the specimen could be directly imaged without the need for additional filtering
implementation, the HS camera would directly capture the image of the flow channel. The baseline spectrum produced by 635 nm laser would be recorded to be subsequently subtracted from the spectrum of obtained data.

After collecting data using alternative imaging approaches, we would proceed to compare the quantity and quality of the acquired results. Comparison would be performed by analysing signal-to-noise (SNR) ratio, dynamic range, and spectral resolution (for HS imaging) of the data obtained with appropriate techniques. The characteristics are assessed in the following manner:

- **SNR** is a parameter utilized to characterized image quality. It indicates the level of the signal compared to the background noise. Conventionally SNR is calculated with equation (2), where $\mu_{sig}$ – average signal value and $\sigma_{sig}$ – standard deviation of the signal [52].

\[
SNR = \frac{\mu_{sig}}{\sigma_{sig}}
\]  

- **Dynamic range** is used to qualify sensor’s ability to adequately image high and low brightness areas in a snapshot [53]. It is an important parameter in our context, since it would give us an estimate of the number of intensity levels possible to distinguish with a particular imaging modality. Dynamic range ($DR$) is calculated as a ratio between the largest ($i_{max}$) and the lowest ($i_{min}$) nonsaturating signals in an image (3). Higher dynamic range indicates enhanced imaging capability.

\[
DR = \frac{i_{max}}{i_{min}}
\]

- **Spectral resolution** is a measure of the ability of the imaging system to distinguish between adjacent wavelengths, which in our case would be a characteristic applied to HS imaging only. It is calculated as the ratio between spectral range and number of imaged spectral bands in the image. For the HS camera we will be using, this parameter can be set up manually.

The measurement setup configuration would also have an influence on the recorded data. Alternative approaches to identify an optimal configuration are discussed in the following Section 3.2.2.
3.2.2. Experimental setup

Optimally, it would be desirable to develop a setup, where excitation and detection components would be located in the same geometrical plane. In the long run this would enable arrangement of the constituent parts in the same submodule and make the obtained system independent of the chosen flow cell design. One possibility of how this could be realised can be seen in Figure 14. In our setup we are going to be utilizing 635 nm emission wavelength only, but the number of utilized lasers can be potentially increased to four.

![Figure 14. One-plane setup configuration, laser illumination coming sidewise.](image)

The built setup can be observed in Figure 15. RBG RGN in mounted on top of the microscope. For the series of tests where HS camera is utilized, it is fixed to the eyepiece. This is done partially due to the lack of mounting component in the employed model of the HS camera. However, having RGB and HS camera simultaneously capture the same image also gives us the advantage of direct comparison between those.
Figure 15. One-plane setup assembled, fluidics and optics subsystem implemented.

Outlook of the interrogation region can be seen in the finer details in Figure 16. The frontal light diffuser is located at the tip of the optical fibre. Since fluorescence has low intensity, it is important to isolate this region of the setup from external light sources to ensure accurate measurements. This can be done by positioning black foil over the setup and turning off the external light sources when performing the tests.

Figure 16. Interrogation zone configuration.

While the one-plane configuration offers the discussed advantages, sidewise illumination might lead to uneven fluorophore excitation and light scattering. This has a potential to generate misleading information regarding particle properties and location in the flow. To
be able to justify how much such arrangement could affect the results, we are going to perform the measurement with an alternative componental layout, so that light source would be located in the opposite plane to the detector (Figure 17). This configuration is similar to the one implemented in traditional optical microscopy and has also been widely utilized in conventional FC.

![Diagram](image)

**Figure 17.** *Two-planes setup configuration, laser illumination positioned opposite to the detector.*

The positioning of the components in this setup would be the same as depicted in Figure 15, with the exception of laser fibre being placed beneath the flow cell. In addition to that, the base plate for the flow cell holder would not be used in this arrangement to allow light to pass through the cell.

### 3.2.3. Hyperspectral image interpretation

Before proceeding with the imaging of the actual particles, let us review a couple of sample images obtained with hyperspectral Senop camera and the procedure of their interpretation. The interface of the camera software reveals us several figures at a time, which include the actual image along with the spectral plot.

The image can either be converted to RGB, or it can be a black and white image. We are mainly going to be working with the latter. For them white would indicate the highest intensity region in the image. Since intensity distribution varies for each wavelength, a particular wavelength must be chosen as “Selected wavelength” to be displayed. The perceived image would in turn exhibit significant variations based on the chosen display wavelength. The impact of this phenomenon can be observed in Figure 18.
The next parameter shown to us is the spectral plot of the selected pixel. The spectrum is positioned to the right from the 2D image (Figure 19). The number of imaged wavelengths is selected manually before the image capturing and is proportional to the imaging speed of the camera. In Figure 19 we can observe that the spectrum would vary depending on the chosen pixel. As discussed in Section 2.2, this is due to the fact that obtained spectrum reflects the properties of the imaged material in terms of its light interaction characteristics.

**Figure 18.** Microscale imaged with hyperspectral camera, illumination with broad spectrum Nikon E400 microscope illuminator. The identical imaged area displayed at a) 900 nm and b) 550 nm.

**Figure 19.** HS snapshot of a laser module. The circles highlight the pixels, which spectra are exhibited to the right. Green reflects the pixel within a laser module case, dark pink reflects the pixel from the table. Both images are displayed at 661.2 nm.
To put it concisely, the spectrum on the right (Figure 19) reflects the selected location on the image, while the image on the left illustrates the state of the screened sample for one selected wavelength at a time. That way, unlike in RGB imaging, where there is just one image reflecting the captured area, in HS there would not be such “general” image. The data would have to be tuned according to the region and wavelength of interest.
4. RESULTS AND ANALYSIS

In this Chapter we are going to investigate the obtained results captured via various imaging modalities. After familiarization with the results, we would then proceed to compare the content and quality of the data achieved with alternative imaging methods. The used beads for all of the performed tests have been described in Section 3.1.3. All images are captured with 20x magnification.

4.1. Non-fluorescent bead imaging

For the calibration purposes we are first going to capture images of the non-fluorescent particles of various sizes. The main goal is to understand how well they can be visualized and distinguished with our setup. For all tests with non-fluorescent particles the wide bandwidth Nikon E400 illuminator will be used as the source of the illumination.

4.1.1. Red-Green-Blue imaging

As we can observe in Figure 20, beads of 16.8 $\mu m$ diameter are clearly differentiated with the utilized imaging assembly. These beads are closer to the size of standard eukaryotic cell, which tend to range from 10 to 100 $\mu m$ in diameter. Non-fluorescent beads of size 3.1 $\mu m$ are also visibly differentiated, however their morphological properties are harder to distinguish at the given magnification. Additionally, due to the relatively big height of the flow channel in relation to the bead size and the lack of focusing, it can be observed that a high fraction of beads stays out of focus. Beads could be clearly distinguished at flow speed up to 20 $\mu l/min$.

![Figure 20. Non-fluorescent beads imaged with RGB camera; bead diameter of a) 16.8 $\mu m$, b) 3.1 $\mu m$.](image-url)
4.1.2. Hyperspectral imaging

For better understanding of HS camera imaging capabilities, let us consider Figure 21, where the same beads arrangement is captured as in Figure 20 (a). 540.5 nm was selected as visualization wavelength as it was found to provide the best resemblance to the RGB camera snapshot.

![Image](image.png)

**Figure 21.** Non-fluorescent beads of 16.8 μm diameter imaged with a) RGB camera, b) HS camera, visualized at 540.5 nm.

It can be noted that the quality of HS camera image is visibly lower due to lower sensor resolution (1024x1024 pixels for HS camera [36] vs. 2592 x 1944 pixels for RGB camera [54]). In addition to imaged particles, HS camera has captured more background details than RGB. In the lower part of HS image, we can note some additional dark regions, which are most likely caused by dust particles contamination. The RGB camera might have not captured those due to differences in its focus area.

However, as we have discussed in Section 2.2, the key benefit behind the HS imaging is the unique capability to get the spectrum of each point in the snapshot along with the ability to extract information from narrow bands of spectrum. In Figure 22 we can observe the spectrum recorded for the pixel, where non-fluorescent particle is positioned, and compare it to the spectrum of the region free from any particles.
Figure 22. Non-fluorescent beads of 16.8 μm diameter imaged with HS camera with spectra of the bead and the channel, a) visualization at 540.5 nm, b) spectrum of empty channel space, c) spectrum of non-fluorescent bead.

The spectral differences are quite drastic, with the peak intensity for flow channel in 630-650 nm region, and for the non-fluorescent bead in 745-830 nm. Since non-fluorescent beads do not produce fluorescence, we can account the obtained complicated spectrum to the light scattering properties of polystyrene. The spectrum measurements have been very consistent throughout the whole image for both: particles and empty channel regions.

4.2. Fluorescent beads imaging

The imaged fluorescent beads are of the same size as seen in Figure 20 (b). Firstly, we are going to estimate if they can be distinguished with the same RGB camera we have been utilizing in prior experiments. Then, we will proceed to imaging those with HS camera and multimode plate reader. The excitation source for RGB and HS imaging will be 635 nm laser. The fluorophore which is expected to be exited at this wavelength is APC-A with emission peak at 670 nm. In the obtained images we are mainly interested in the distinguishability of excited particles, possibility to identify the fluorophore via its spectral footprint and possibly differentiate the particles based on the recoded level of fluorophore intensity. The excitation laser is positioned sidewise unless otherwise specified.

4.2.1. Red-Green-Blue imaging

As seen in Figure 23, fluorescent beads can be distinguished clearly with RGB camera even without introduction of additional excitation wavelength filtering for reducing background noise. The power of the excitation laser has been increased gradually to observe changes in the recoded snapshots. At 20 mW (Figure 23 (a)) we can note that
the background laser noise level is still in the same range as the recorded light and the camera software is not capable to fully differentiate and suppress it. However, for higher power levels (Figure 23 (b, c)) the fluorescent particles can be more easily visibly differentiated, and the background noise is filtered out.

![Image](image.jpg)

**Figure 23.** Fluorescent beads excited with 635 nm laser at a) 20 mW, b) 50 mW, c) 100 mW power; all images recorded with RGB camera.

At 100 mW the particles appear overly bright, which could be possibly contributed to light scattering and camera detector saturation for higher power levels. This might be undesirable since it can distort the actual fluorescent pattern. Traditionally, the used power level for excitation lasers in flow cytometry is 50 mW (as discussed in Section 3.1.2). In our further tests it is going to be used as well, since it has been shown to provide good results without overexciting the particles.

An additional point to note about fluorescent beads images is that all particles despite their height in the flow channel are recorded simultaneously. In the meanwhile, it was different for earlier experiments with brightfield illumination, which is used commonly in microscopic imaging. There we have managed to record only a small fraction of particles in focus at the same time.
4.2.2. Hyperspectral imaging

Let us proceed to capture the fluorescent particles arrangement in the flow channel with HS camera (Figure 24). It was empirically deduced that the best image visualization was achieved at 630 nm (as seen in Figure 24 (a)). Visualization of fluorescent particles at other wavelengths has failed to produce image with clearly distinguishable elements.

![Figure 24. Fluorescent beads excited with 635 nm at 50 mW power. Data captured with HS camera; a) snapshot visualized at 630 nm, b) spectrum of empty flow channel space, c) spectrum of fluorescent particle. For (b) and (c) 650 nm wavelength is marked with black line for clarity.](image)

Pixels within the fluorescent particles’ regions were observed to produce similar-looking spectra with intensity peak at 626 nm. In the meanwhile, the intensity peak of the background stayed around 638 nm, which corresponds to the excitation laser spectrum. While fluorescent beads have been shown to be clearly differentiated in terms of recorded spectrum, the results are counterintuitive, since the emission for APC-A is expected to take place at 670 nm. It could be possible that crosstalk between various fluorophores or aberrations in the light path could have accounted for the spectrum shift.

Despite the wavelength shift, with this visualization method it was possible to differentiate between various fluorescence intensity levels. As it was discussed, the utilized particles have in total eight intensity peaks. In our setup it was possible to differential between at least three different levels. In Figure 25 the obtained intensity (expressed radiometrically for the given image) is given for three fluorescent particles, differentiation between which would have been cumbersome with the naked eye.
Figure 25. Fluorescent beads intensity levels variations, a) HS snapshot, visualized at 630 nm, b) background laser noise with intensity peak around 0.2 a.u., c-e) fluorescent particle with intensity peaks around 0.23 a.u., 0.31 a.u., 0.48 a.u. respectively.

4.2.3. Plate reader imaging

To compare the images obtained from our tests with a reference, the same beads solution was imaged with a multimode plate reader. While it also implements RGB camera for image capturing, the key difference between our RGB snapshot is that multimode plate reader introduces filtering of the excitation light. The obtained image (Figure 26) should thus be free of background excitation source noise and only feature the fluorescent light emitted by the beads.
Figure 26. Plate reader snapshot of fluorescent particles, excitation with 632 nm LED with Full Width Half Maximum (FWHM) = 20 nm; emission recorded at 708 nm with FWHM = 89 nm.

The broad band of emission recording, however, might still enable crosstalk with other fluorophores present in the solution. Which is nonetheless less likely due to APC-A being the brightest excitable fluorophore in the studied part of the spectrum. Although the intensity levels of the beads in the captured image can be distinguished to some degree, the center of the recorded emission peak is at 708 nm, which is suboptimal given that the peak of the APC-A fluorophore emission is at 670 nm. If these two peaks had matched, the brightness of the particles in the image would have been about twice as high.

4.2.4. Upward laser illumination

It was originally assumed that upward illumination might offer benefits over sidewise in terms of illumination uniformity. On the contrary to this assumption, upward laser illumination was shown to be much less effective for our setup than sidewise illumination (Figure 27). This can be mainly contributed to saturation of the detector sensor with the high-power laser aimed directly at it. The laser power level was decreased to as little as 1 mW, however the image remained overexposed.
Thus, even at lower laser powers the quality was still inadequate, and the particles were too hard to differentiate. The same was applied to HS imaging, where it was not possible to produce an illustrative image for this geometrical arrangement.

4.3. Results discussion

For brightfield illumination tests, despite lower sensor resolution for HS camera, more image details were seen to be differentiated in the snapshot made with it (Figure 21). By visualizing surrounding particles, which may have been dust on the flow cell, we can obtain information not only about the plane in focus, but also about the imaged surroundings. This is particularly valuable as it allows us to identify possible contamination sources, which could potentially affect the recorded data.

The obtained signals from each of the recording modalities when excited with laser (RBG and HS cameras) or red LED (plate reader) are summarised in Figure 27. While particles can be differentiated in all of the images, the data content in each of them varies.
Let us proceed to compare SNR and DR of the snapshots utilizing the equations described in Section 3.2.1. To obtain the signal values, we first have to convert the images to grayscale. It is required for direct comparison between black-and-white and colourful snapshots. Then, we should find the average value of the signal and its standard deviation as 8-bit pixel values. This parameter comes from the fact that each pixel occupies exactly one byte and can be assigned $2^8$ or 256 numerical values, from 0 to 255. The appropriate ratios of 8-bit pixel values define SNR and DR. The described processing steps are performed with MATLAB software. SNR is found as a ratio of average signal value to background noise. For its determination the following script can be utilized:

```matlab
img=imread("Image_name.png"); %Reading file
img=double(img(:)); %Type conversion from integer to double
ima=max(img(:)); %Finding the brightest image point
imi=min(img(:)); %Finding the dimmest image point
ims=std(img(:)); %Calculating std
snr=10*log((ima-imi)./ims); %Applying formula(2)
```
DR is found as a ratio of the brightest recorded particle intensity value to the least bright. With MATLAB it can be found as following:

```matlab
max = max(max(img)); %Brightest image pixel value
min = min(min(img)); %Dimmest image pixel value
dr = max/min; %Applying formula (3)
```

As discussed, DR defines the possibility to distinguish between different intensity values in the recorded signal.

The summary of the calculated results is given in Table 1. The average of the results from three different recordings was utilized to define SNR and DR in each imaging type. Relatively low DR can be contributed to limited bit depth, which leads to brightness levels being defined with bit values close to each other.

**Table 1. SNR and DR for the utilized imaging modalities.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RGB imaging</th>
<th>HS imaging</th>
<th>Plate reader imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signal-to-Noise Ratio</td>
<td>33</td>
<td>21.2</td>
<td>18.5</td>
</tr>
<tr>
<td>Dynamic Range</td>
<td>1.3</td>
<td>2.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

From Table 1 we can conclude that the best SNR was achieved in snapshots captured with RGB camera. Multimode plate reader has been shown to be the noisiest one of the three, which can be contributed to plate reader having wider excitation bandwidth, which could have potentially excited other fluorophores than APC-A with close excitation regions. This could have led to extra noisy background.

In terms of DR, HS imaging has demonstrated the best results. Thus, when analysing the possibility to differentiate between various intensity levels within a single snapshot, HS imaging has demonstrated a more extensive ability to do that. When regions of RGB or multimode plate reader images containing imaged particles were converted to greyscale, the obtained values were too close to conclude on the presence of various intensity levels. However, even for HS imaging where the difference in the intensities is demonstrated with higher DR, the nature of this difference has yet to be confirmed. Various intensities could either originate from different intensity levels of the fluorophore markers present in the sample solution or they could be caused by particles being located in different imaging planes within the flow channel.
The spectral resolution was varied from 20 to 50 bands for the set of experiments utilizing hyperspectral camera. These numbers were sufficient to read the spectrum of the image, however it was identified that the duration of all snapshots acquisition was not optimal. This is due to nature of frame-based snapshot imaging, and the fact that going through all of the bands one by one added up to several seconds for one image acquisition. The fluid, thus, had to be kept stationary when imaged with HS camera. If this imaging mode is to be further implemented to flow cytometry, then snapshot imaging, where all bands are captured simultaneously, should be preferred to frame-based snapshot.

The fact of fluorophore markers identification with the HS camera is yet to be confirmed. Even despite APC-A being considered a bright fluorescent marker, it could still be possible that laser light intensity exceeded the dynamic range of the fluorophores. This could have then resulted in fluorophores being indistinguishable from the background light. If that was the case, then the spectral shift in the recorded light could be explained by the recorded light being the scattered laser light and not the fluorescence. To further confirm this, it would be interesting to conduct the same experiment with fluorophore particles excitation with laser wavelengths alternative to 653 nm. If the recorded spectrum of the particles would be close to the original excitation wavelength, then the signal originating from laser light scattering would be the most probable scenario. If the fluorophore spectra would correspond closer to the theoretical ones, then the spectral shift origins in our experiment would need to be further investigated.

Alternatively, it would be feasible to employ band-stop filter to see if the dynamic range of the obtained image would then be suitable for fluorophore distinguishment. Originally, the implementation of additional filters was discouraged since that would have overcomplicated the system. Particularly, in the situations where multiple laser wavelengths were to be implemented, filter change would need to be established. Theoretically, HS camera should present information on all of the present wavelengths, and filtering out laser illumination wavelength could have been done later at the data processing step. But if fluorophore intensity is not high enough to be recorded in this manner, filtering approach could be tried to determine its efficacy. It could also lead to better particle differentiation in the setup arrangement with upwards laser illumination.
5. CONCLUSIONS

In the scope of the performed work, the applicability of hyperspectral imaging to the flow cytometrical applications was researched. Potentially HS camera could increase the information content of the gathered cytometrical data and replace the complicated optical arrangement of detector arrays employed nowadays with a single HS camera. Based on the results, it was concluded that this approach shows great promise in upgrading the existing imaging flow cytometry technology. However, there remains a number of points for future investigation.

The aim of the thesis was achieved by performing a series of experiments to evaluate HS applicability to flow cytometry in a comprehensive manner. It was experimentally demonstrated that based on the spectrum recorded with HS camera, the particles could be easily differentiated from the sample flow. The nature of the recorded spectrum for the fluorescent particles is yet, however, to be further researched. Implementation of filtering for the excitation laser light would be a possible approach to identify if the fluorescence emission can be better visualised in the absence of the excitation light oversaturating the signal. Side illumination was identified as the preferable geometrical arrangement in the absence of additional filtering.

To be able to better differentiate between the recorded intensity levels and justify on their nature, improvements to the fluidics system could be implemented. Those can either include introduction of a focusing method, such as hydrodynamic focusing, or a modification of the utilized flow cell design. In particular, the height of the flow cell could be decreased to avoid formation of multiple particle planes, which could complicate the analysis.

Among the possible paths for future research, one could consider confirming the ability to visualize fluorescence signal in the recorded HS snapshots. In addition, it would be valuable to confirm the possibility to differentiate the localisation of the fluorophore within the imaged cells by conducting HS imaging tests on the labelled cell culture. If proven to be effective, the method holds great potential for improving the efficiency of disease diagnostics, among other applications of biomedical imaging.
REFERENCES


