Mass cytometry is one of the newest and most high-throughput technologies that allows for the investigation of complex biological systems on single cell level. At the beginning, mass cytometry was mostly used for the analysis of different cell populations present in various biological samples. Now, after years of development, this technique grants in-depth analysis of whole proteomes within complex samples, such as cancer tissues. Mass cytometry relies on the use of stable metal isotopes as labels of specific cell markers and therefore, allows for simultaneous analysis of more than 40 parameters on single cell level. In order to fully explore the potential of mass cytometry, researchers all over the world are trying to develop new experimental setups based on the application of pure metal isotopes in biological studies. The incorporation of antibodies into mass cytometry setups, while extremely selective and well-validated, limits the analysis as it shows the whole protein pool present in the cell. In the presented doctoral dissertation, I have developed new technology that allows for the identification of active forms of proteins-the ones that actively participate in cell signaling pathways. I have designed and synthesized small molecule chemical markers labeled with stable metal isotopes and subsequently used them to determine the activity status of individual enzymes. Activity-based probes are the most valuable tools for enzyme activity profiling and for years now they have been in the center of the method called Activity-Based Protein Profiling. Classic activity-based probes consist of three parts: a warhead (electrophilic binding group that covalently modifies enzyme active site), linker (specific peptide sequence or non-specific carbon chain) and the fluorescent tag that allows for enzyme detection and localization inside the cell. Spectral properties of commercially available fluorophores allow for the detection of up to dozen different cell parameters, with the use of various techniques such as confocal microscopy or flow cytometry. In order to increase the number of analyzed parameters, I have designed activity-based probes that possess DOTA chelating moiety instead of fluorophore. This chemical moiety is able to trap one metal atom per one probe. The combination of mass cytometry with highly selective activity-based probes allowed for the development of new technology that grants the possibility of multiparametric analysis of complex biological samples. The presented dissertation is focused on the analysis of proteolytic enzymes involved in cancer development and progression, such as cysteine cathepsins, caspases and neutrophil serine proteases. The new type of activity-based probes (so-called TOF-probes) presented in the dissertation incorporate various inhibitor scaffolds designed with HyCoSuL technology (*Hybrid Combinatorial Substrate Libraries*). These compounds possess a variety of unnatural amino acids in their structures, which significantly increases their selectivity toward proteases of interest. Such inhibitors were labeled with stable metal isotopes, thus creating a panel of TOF-probes for protease activity analysis within the cell. What is more, with the use of designed compounds and the newest imaging system called Hyperion, I have shown the spatial distribution of active cysteine cathepsins within cancerous cells. The TOF-probes were used simultaneously with metal-tagged antibodies in order to identify cell subpopulations and also, to determine the total protein amount (active and not active forms). Presented technology for labeling active proteases was thoroughly validated. I have performed a full range of independent experiments, ranging from kinetic analysis on

pure, recombinant enzymes, through comparison of fluorescently labeled activity-based probes with newly synthesized TOF-probes, up to biological experiments where the total protein amount was correlated with active portion of targeted proteases. Obtained results allowed to determine the level of protease expression (antibodies) and activity (TOF-probes) in selected cell lines and peripheral blood mononuclear cells. I have discovered that the activity status of individual enzymes is not uniform across different cell populations and, what is more, the activity of the enzyme does not always correlate with its expression level. The presented doctoral dissertation shows the first application of small molecule activity-based probes in the analysis of the functional state of proteins with the use of mass cytometry. Moreover, developed technology can be further used to study a variety of medically important enzymes such as kinases or phosphatases. The activation of individual proteins within the cell brings information about cell responses to different stimuli from inside and outside the cell. This in turn is absolutely necessary to determine cascades of activation of cell pathways that can lead to pro-health events and also, to pathophysiological conditions. Designed experimental protocols presented in the dissertation allow for precise analysis of enzyme functional state in various cell populations whereas the use of mass cytometry enables multiparametric analysis of these phenomena.