Summary
Proteins are complex biomolecules which are involved in a wide array of functions, from cytoskeletal structure to immune system, enzymes to hormones, and nutrition to medicine, which makes them a vital component of the life. Any change in the basic structure or composition of the protein might alter the physiology of the cell and tissues and eventually may lead to pathological conditions or diseases. Hence, the study of these biomolecules holds a great deal of importance in understand and maintaining homeostasis of every cellular machinery. Presently available techniques carryout the purification, separation, identification and analysis of proteins in different stages and there are no techniques for the quick assessment of this biomolecule under single platform. In this context we have developed a technique by integrating the fluorescence spectroscopy with 1 and 2D gel electrophoresis for stains- and tag-free visualization, differentiation, analysis and identification of proteins on a single platform. The technique can also be coupled with instruments like mass spectrometry and Western blot if needed for the quick assessment of proteins.

In the present work intrinsic fluorescence spectroscopy was integrated with 1 and 2D-poly acrylamide gel electrophoresis (PAGE) and autofluorescence spectral recording conditions were standardized in three different steps. Step one was the development of laser induced autofluorescence based instrumentation for recording autofluorescence of proteins from polyacrylamide gels. Step two was the standardization of methodology for recording fluorescence of proteins in PAGE and step three was the development of autofluorescence based PAGE fingerprint using MATLAB algorithms for visualization, differentiation, analysis and identification of proteins.

Laser coupled automated X-Y translational stage based instrumentation for autofluorescence spectral measurements of proteins in PAGE
The excitation of 281 nm light was obtained from second harmonic Nd-YAG laser pumped frequency doubled dye laser (dye source-Rhodamine 6G) system. The in-house fabricated sample-holding platform was rigidly fixed over a micro-controlled automated XY translational stage. The sample holding platform was designed to host either 96-well plate or 10cm x 10cm x 1mm quartz plate. The 96-well plate was used for fluorescence recording in the aqueous phase and quartz plate was used for fluorescence recording from PAGE gels. The fluorescence signals were collected using a telescopic arrangement of lenses and filter
and carried to the spectrograph-ICCD using optical fiber for spectral dispersion and detection. The microcontrolled automated translational stage provided a precision guided movement across the PAGE gel against fixed laser beam of light to find the protein spots in unstained and untagged gel.

**Novel spectral recording methodology for the autofluorescence based interrogation of proteins in PAGE**

The relationship between aromatic amino acids and their autofluorescence properties was evaluated using HSA as a model protein which demonstrated that fluorescence properties of tyrosine and tryptophan in congruence can be used to assess the structure and microenvironment of protein. As a step towards the standardization of autofluorescence recoding proteins in PAGE, the effect of all the chemicals such as detergents, chaotropic agents and reducing agents and protein denaturation conditions used in PAGE were tested against the autofluorescence properties. The chemicals found to quench or alter the autofluorescence properties were removed, replaced or decreased to negligible levels to overcome the effect of those chemicals. During the study some important observations were made:

SDS interacts differently with different proteins based on their structural complexity. It induces hydrophobic collapse around tryptophan residues in HSA and BSA. However, it results in the complete unfolding of trypsin but has no effect on the autofluorescence properties of lysozyme, CA and RNase A. This indicates that the change in the emission wavelength in the protein is greatly influenced by the structural complexity of the individual proteins. The interaction of proteins with chaotropic agents demonstrated that the emission shift in protein not only depends on the structural complexity of the protein but also on the ratio of tyrosine to tryptophan in them.

Tetramethylethylenediamine (TEMED) and acrylamide were found to quench the protein fluorescence. Since acrylamide and TEMED are dynamic quenchers the un-polymerized and unreacted molecules were removed by extensive washing with distilled water. The quantity of the TEMED used for the polymerization was reduced from 0.3 to 0.1 mM. The protein fixing agent was found to give background spectra at 350 to 450 nm region; hence, the step was postponed after fluorescence recording from unstained and untagged PAGE gel.
Chapter 7.

The present study has demonstrated the influence of reducing agents used in the PAGE on the autofluorescence properties of proteins. The sulfur containing disulfide bond breaking agents such as BME and DTT showed fluorescence quenching. Redox reactions in the excited fluorophores of the proteins in presence of BME showed unusual red shift and quenching of the corresponding fluorescence because of photo induced PCET. However the phosphorous containing disulfide bond reducing agent TCEP was found to have no influence on the autofluorescence properties of proteins and hence was used as alternative for BME and DTT in PAGE experiments. With the implementation of all the changes in PAGE methodology based on the above observations we recorded a minimum of 19 ng of CA using TCEP-SDS PAGE in contrast to 78 ng of CA in conventional BME-SDS PAGE, suggesting higher sensitivity of TCEP over BME.

Fluorescence spectra recorded for seven different pure proteins in PAGE gave fluorescence intensity and emission wavelength based on the number and distribution of fluorophores in the protein. The emission wavelength was observed specific for each of the proteins under the standard conditions discussed in the present work and demonstrated distribution of over 36nm range for RNase A (313 nm), HSA (318 nm), BAS (325 nm), trypsin (335 nm), carbonic anhydrase (337 nm), lysozyme (340 nm) and cellulase (354 nm). This gave us a clear idea that the specific emission wavelength of the protein based on its structure and composition can be a useful tool for the identification and analysis of protein in PAGE. Keeping the information obtained from the PAGE such as MW and pI we tried to represent the fluorescence intensity and emission wavelength of each protein spot on a single platform. This would give us scope to compare multiple proteins based on four different variables such as MW, pI, fluorescence intensity and emission peak. To accomplish the same we developed the autofluorescence based PAGE fingerprint of proteins using the MATLAB based algorithm.

**Matlab assisted software design for the development of PAGE fingerprint**

MATLAB assisted software was designed to develop PAGE fingerprint using autofluorescence intensity and peak wavelength values of protein spots in PAGE. The software shows the protein spots in the form of pixels. Inside each pixel, intensity values have been realized in the form of a grayscale image whereas the wavelength values were realized in the form of colors and shapes of different denominations. In an experiment using
pure proteins we demonstrated ability of autofluorescence based fingerprint to identify the overlapping and co-migrating proteins based on two different parameters. One is based on the emission wavelength represented as specific color and shape (Fig. 5.9) in the fingerprint and second is based on the additive, synergistic and antagonistic autofluorescence intensity of overlapping proteins which is understood with help of RFI calculation (table 5.1).

The software has the ability to readjust the size and dimensions of the fingerprint according to the exact size of the original PAGE gel. This makes it easier for spot-picking for mass spectrometry without staining or tagging. The mass spectrometric validation of the protein spots eluted from the 2D PAGE gel based on the emission wavelength and fluorescence intensity/density ratio demonstrated that proteins having the ratio of tyrosine to tryptophan more than 3:1 have shown blue shifted emission and in the present study they come under the blue emission region of fingerprint (317-324 nm). At the same time proteins with tyrosine to tryptophan ratio less than 3:1 have shown emission between 325-335 nm which in the present study comes under green and yellow emission regions of the fingerprint. Mass spectrometry results were in agreement with our earlier results that the proteins show the huge blue shift when they have high tyrosine to tryptophan ratio as well as their tryptophan residues undergo hydrophobic collapse.

**Major findings**

1. For the first time, the methodology has been standardized for recording intrinsic fluorescence of proteins in PAGE.

2. The instrumentation and optimized methodology for recording of autofluorescence of proteins in PAGE and the Matlab assisted software when combined together, provided a sensitive tool for stain- and tag-free visualization of protein spots in 1D and 2D PAGE.

3. The present technique has demonstrated a 19 ng protein band identification which is more sensitive than the coomassie staining.

4. The study delivers autofluorescence based protein fingerprint in PAGE and gives scope to interrogate proteins by providing the information of intrinsic fluorescence intensity, emission peak, molecular weight and an isoelectric point on a single platform.
Chapter 7.

5. The technique can differentiate overlapping protein spots which otherwise cannot be differentiated in conventional staining, imaging and tagging methods, providing an opportunity to study protein-protein interactions in PAGE.

6. As one of the possible applications for PAGE fingerprint, we have demonstrated the identification of tyrosine containing and tryptophan missing proteins.

7. Since, there is no interference by stains or tags in the methodology, the present technique can also be coupled with liquid chromatography and mass spectrometry for initial screening of proteins and spots selection.

Future perspectives
For the first time, an effective methodology for the recording of autofluorescence properties of proteins in PAGE has been standardized. This opens up the door for many of the absorption and emission based techniques to directly record such information in PAGE such as fluorescence lifetime, decay, fluorescence resonance energy transfer (FRET) and Fluorescence recovery after photobleaching (FRAP) etc. The emission wavelength and intensity have shown direct correlations with the structural complexity of the native proteins and hence this technique can have applications in studying protein foldings. The developed method not only helps in the differential identification of overlapping or co-migrating proteins but also may give a clear indication when proteins overlap, bind or come in contact with other proteins, ligands or metal ions that can be detected. Hence, the technique can be useful in the study of protein-protein interactions such as antigen-antibody reactions without the need for Western blotting.