Claims

1. A method of antigen retrieval of a fixed sample comprising the steps of:
   a) incubating the fixed sample in a first antigen retrieval solution at a temperature between 50 to 75 °C;
   b) transferring the fixed sample to a second antigen retrieval solution; and
   c) incubating the fixed sample in the second antigen retrieval solution at a temperature between about 50 to 75 °C,

   wherein the first antigen retrieval solution comprises a buffer solution having a pH range of between about 5 and 7 and the second antigen retrieval solution comprises a buffer solution having a pH range of between about 8 and 10; or

   the first antigen retrieval solution comprises a buffer solution having a pH range of between about 8 and 10 and the second antigen retrieval solution comprises a buffer solution having a pH range of between about 5 and 7.

2. The method according to claim 1, wherein the fixed sample is a biological sample, preferably comprising biological tissue from human, animals, or plants, further preferably comprising biological cells and/or extracts and/or part of cells.

3. The method to any one of claims 1 to 2, wherein the fixed sample is a frozen-fixed tissue sample, in particular a cryosection of a tissue.

4. The method to any one of claims 1 to 3, wherein the sample is fixed in crosslinking fixatives.

5. The method to any one of claims 1 to 4, wherein the sample is fixed in coagulant fixatives like alcohol, and acetone.

6. The method to any one of claims 1 to 5, wherein the sample is fixed in formaldehyde and embedded in paraffin (FFPE), in particular a cryosection of a formaldehyde-fixed biological sample.

7. The method to any one of claims 1 to 6, wherein the sample is a frozen fixed tissue sample and post-fixed with coagulant fixatives like alcohol or acetone and/or with crosslinking fixatives like formaldehyde.

8. The method according to one or more of the above claims, wherein the fixed tissue sample undergoes *in-situ* hybridization analysis.

9. The method according to one or more of the above claims, wherein the fixed tissue sample undergoes FISH or CISH analysis.

10. The method according to one or more of the above claims, wherein the fixed tissue sample undergoes spatial biology analysis like spatial transcriptomics and/or spatial proteomics.

11. The method according to one or more of the above claims, wherein before the antigen retrieval the fixed tissue sample is analyzed by an *in-situ* hybridization method and after the antigen retrieval the fixed tissue sample is analyzed by a immunohistochemical staining of the antigens.

12. The method according to one or more of the above claims, wherein after the antigen retrieval the fixed tissue sample is analyzed by an *in-situ* hybridization method and/or by an immunohistochemical staining of the antigens.

13. The method according to any one of claims 1 to 12, wherein the analytes are fixed in a permeabilized sample, such as a cell-containing sample.

14. The method to any one of claims 1 to 13, wherein one or more of the sample handling is automated.

15. A method according to any of the proceeding items for the use in an application selected from the group consisting of identify regions of interest, for image analysis, develop cell segmentation algorithms using immunofluorescence images, to aid the segmentation of cells in a sample, to establish AI-based image analysis algorithm using immunofluorescence images to identify disorders, feature extraction like nucleus morphology, signal homogeneity, signal intensity and/or centroids, to determine expression of proteins and genes of interest via semi-quantitative approaches, diagnosis and prognosis applications, determination of the distribution of a target protein in the of health and disease organ, tissue and/or cell, to predict the prognosis of diseases by using disease-related prognostic and/or predictive biomarkers, to provide disease diagnosis e.g. tumor of unknow origin, neurodegenerative disorders, muscle

disorders, brain trauma, infectious diseases, to predict a therapeutic response, subclassification of diseases, examine gene expression and to study gene regulation in normal and pathological tissues, morphological studies, recognition of tissue structures and/or cell type classifications, multiomics studies, quantitative correlation and spatial colocalization study between ISH and ICH, study epigenetic modifications and chromatin structures, identify spatial distribution, and cell-specific ISH signals (Cell segmentation) and determine the correlation of genetic aberrations and/or the copy number variations (CNV) to the corresponding proteins.