Publishable final activity report

Basic facts

Project title:	Enhanced ligase-based histochemical techniques
Project acronym:	ENLIGHT
Project type:	STREP
Starting date:	August 1, 2006
End date:	July 31, 2009
Duration in month:	36
Keywords:	Cancer biology and diagnosis, biomarker, *in situ* analysis, technology development, proximity ligation assay, PLA, padlock probing
EC contribution:	€ 2 978 810

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Introduction

In situ analysis of cells and tissues has for many years been an essential part of pathological research and diagnosis primarily within cancer, and a number of specific biomarkers of predictive and prognostic value for various cancers have been identified. *In situ* analysis of proteins is dominated by immunohistochemistry where sections of tissues are tested for the presence of proteins by specific antibodies, while *in situ* analysis of nucleic acid sequences is dominated by *in situ* hybridization. Due to limitations in these technologies there is a significant need for new efficient techniques and procedures for more advanced analyses. A major challenge is to develop improved means for more detailed studies of biomolecules *in situ*, in order to determine their abundance, sub-cellular localization and secondary modifications, as well as how they interact with other molecules and participate in signalling and control of cellular function.

Project objectives

The main purpose of the ENLIGHT project was to develop new analytical procedures with the sensitivity and specificity required to study individual nucleic acid and protein molecules and their functional status (interactions and posttranslational modifications) in their normal context in single cells and tissues ("*in situ"*) and on tissue lysate microarrays. The project was based on two fundamental recent technological inventions, the "proximity ligation assay" technology (also called PLA) for protein analysis and "padlock probing" for DNA analysis. These are the first technologies to offer the sensitivity and specificity required for studies of single bio-molecules in single cells and tissue *in situ*.

The detection by microscopy of characteristic biomarkers in cancer cells is based on specific staining of tissue sections or cells isolated from patients and mounted on glass slides. The staining results in coloured or fluorescent signals at those locations where the biomarkers are expressed, and the number and/or intensity of the signals often reflects the

presence (diagnosis), severity (prognosis) and preferred treatment (prediction) of the disease. The quantification of high numbers and varying intensities of signals is very cumbersome (if possible) without assistance from computers and dedicated software programs. A second ENLIGHT objective was therefore to develop automated image analysis procedures to complement the molecular methods, and to achieve quantitative information about what molecules or molecule complexes are present in the sample and their tissue or sub-cellular localization (i.e. in which cellular substructures).

The third objective was to apply the new molecular methods and image analysis procedures to clarify the role of molecular biomarkers in tumourigenesis, primarily concerning the AP-1 and HER protein families and for mitochondrial DNA.

Results

The ENLIGHT project provided novel molecular and software tools to the scientific community in order to study protein and DNA biomarkers in cultured cells and clinical specimens with a higher resolution than possible before the project. More specifically, we developed a generic PLA method for detailed studies of protein biomolecules *in situ*. We explored and optimized the method in order to analyze individual proteins, interactions between proteins, post-translational protein modifications and the sub-cellular localization of these molecules. We furthermore used the padlock probe technology to distinguish single-nucleotide differences between different mitochondrial genomes, and their localization *in situ*. Relative to state-of-the-art procedures, we have shown that these methods can provide significantly improved *in situ* analyses. We furthermore developed automated image analysis procedures to complement the molecular methods, in order to achieve quantitative information about what molecules or molecule complexes are present in a sample and their tissue or sub-cellular localization (i.e. in which cellular substructures). Finally, we generated important new biological knowledge on the role of several biomarkers in tumourigenesis and mitochondrial diseases thanks to the novel molecular methods and image analysis procedures developed in the project.

Uppsala University (partner 2a) invented the PLA principle before the ENLIGHT project started. During the project, the Uppsala lab explored new ways to use PLA. To improve the usefulness of the technology and to facilitate technology transfer to other laboratories, the possibility to use secondary species-specific proximity-probes were explored by Uppsala University in the ENLIGHT project, omitting the need of covalent conjugation of oligonucleotides to each new pair of antibodies (see Fig 1). Modifications of the methods enabled readout by flow-cytometers, bright-field microscopy and the method was shown to have advantages in high-content drug screening. Further improvements of the method was made to allow multiplexed detection (Fig 2).

Partner Olink AB developed reagents and basic PLA protocols for analysis of various oncogenic proteins and protein interactions in cells and tumour tissue samples. These reagents were tested and PLA protocols were optimized in order to achieve robust and functioning reagents. The PLA procedure was furthermore optimized in terms of workload needed, but also in order to improve reproducibility and robustness of the method (i.e. important factors for research and clinical applications). Olink also evaluated antibody reagents provided by Dako and IMS for the use in PLA assays. Some of these antibodies proved to work well in combination with PLA. These assays are included in the assay database now available on Olink's home page.

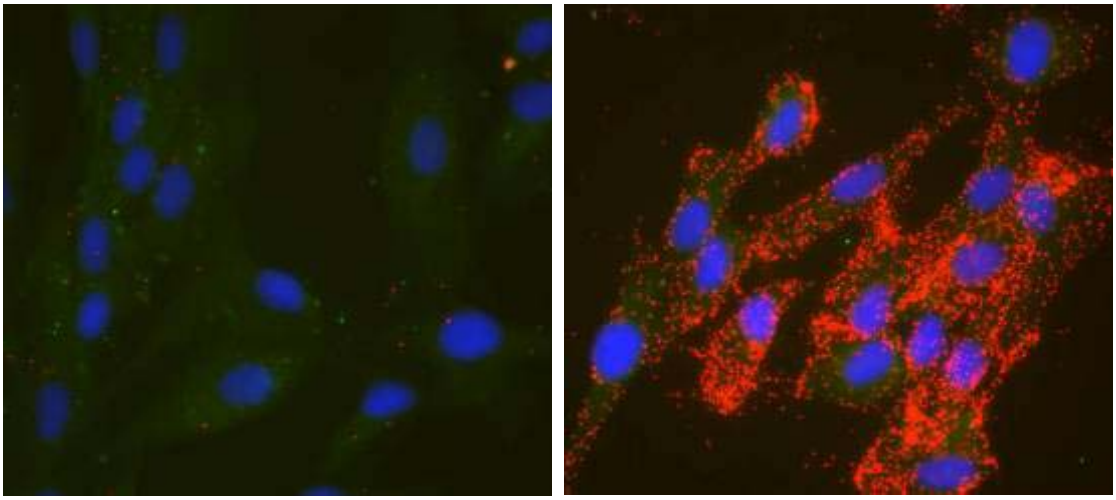

Figure 1. *In situ* PLA for detection of PDGFR-β-phosphorylation in serum starved fibroblasts (left panel) and PDGF-β-stimulated fibroblasts (right panel). Each red dot represents the detection of a single phosphorylated PBGF-β receptor. The cells were counterstained with FITC anti-Actin (green) and Hoechst (blue) to visualize the cytoplasm and nucleus. A dramatic increase in the number phosphorylated receptors as a consequence of PDGF stimulation can be seen (right panel). The number of detected receptors can be automatically counted by new algorithms developed in the project.

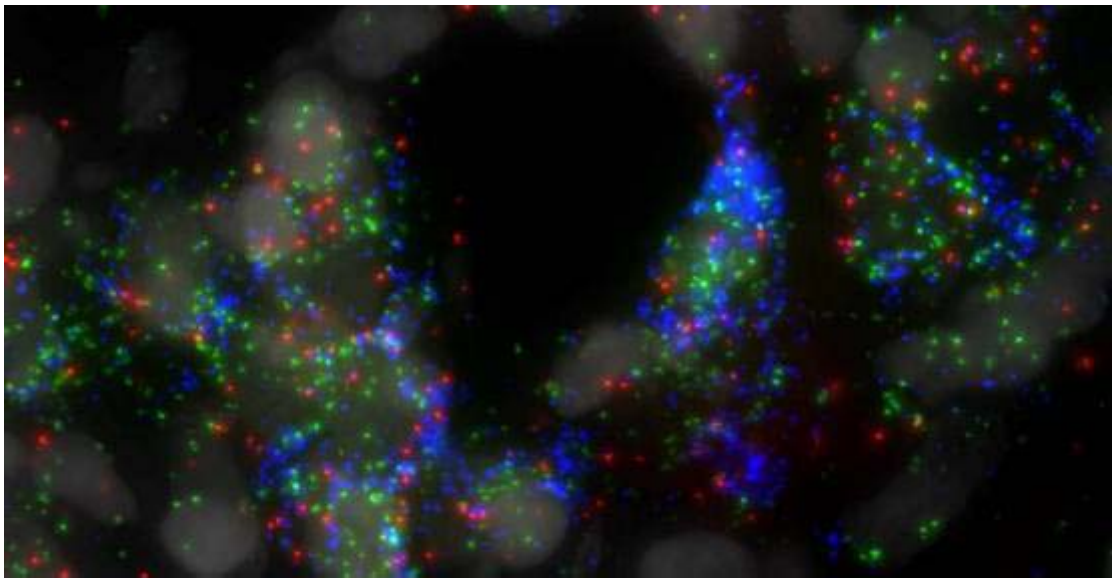

Figure 2. Multiplex *in situ* PLA showing the localization of Human Epidermal growth factor Receptor (HER) dimers in a breast cancer tissue section. Blue dots show single HER2:HER2 homodimers, green dots are HER2:EGFR heterodimers and red dots represent HER2:HER3 heterodimers. A clear difference between cells can be seen. The number of detected dimers can be counted by new computer algorithms developed by the project.

DakoDenmark A/S and ImmunSystem AB provided antibody reagents to the other partners. Antibodies from Dako's catalogue (e.g. pAKT, HER1-4) were offered for technology evaluation and applied studies. Dako furthermore developed and purified several antibodies for the other partners. IMS developed a complete workflow for delivery of polyclonal chicken antibodies, involving selection of suitable target peptide(s), synthesis and purification of peptides, preparation of immunogen, immunisation, crude purification of the antibodies, affinity purification and verification of antibody products. 27 antibodies against candidate tumour biomarkers were developed and tested for use in an immunoassay set up with PLA.

A second research group from Uppsala University (partner 2b) developed new software tools for analysis of PLA and padlock signals and their localization in cells and tissue. Completely new algorithms for quantification of co-localized signals were investigated. A novel image filtering technique that reveals the exact 3D localisation of PLA signals (see Fig 3) was developed and applied to a study of signal translocation in collaboration with partner 2a. Uppsala University furthermore developed new techniques for handling signals from fluorescence multiplexing. The final contribution project is a software, called BlobFinder, that facilitates quantification of PLA signals imaged by fluorescence or classical bright field microscopy. By making the PLA technology compatible with enzymatic signal detection it can be combined with conventional histological staining, commonly used in clinical settings.

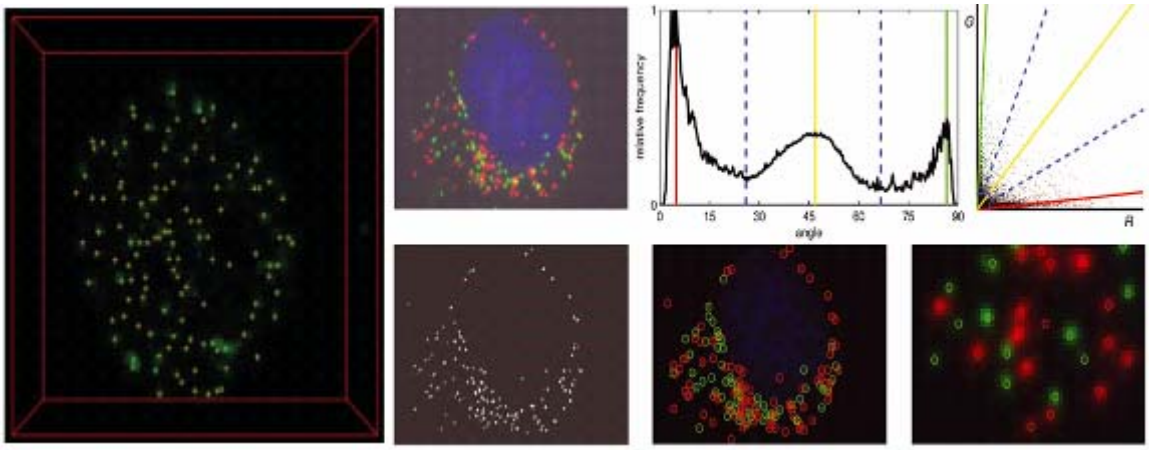

Fig 3. Left: 3D localisation of padlock signals within a cell nucleus. Right: A cell nucleus (blue) surrounded by red and green padlock signals from two types of mitochondrial DNA. The angle histogram shows the distribution of fluorescence in the image with the automatically detected decision thresholds, and the bottom row shows the result of spectral decomposition and classification of detection evens.

The "VIS" software developed by Visiopharm systematizes the collection of PLA image data, and reduces the manual and monotonous imaging work. In ENLIGHT, algorithms for single cell detection (first explored by UU-CBA) were integrated with the VIS-based functionalities allowing automated segmentation of relevant cells and quantification of their PLA signals. The original digital images, the derived metadata achieved by image segmentation, and the final quantitative results obtained by image analysis can all be organised in the dedicated VIS database (Fig 4).

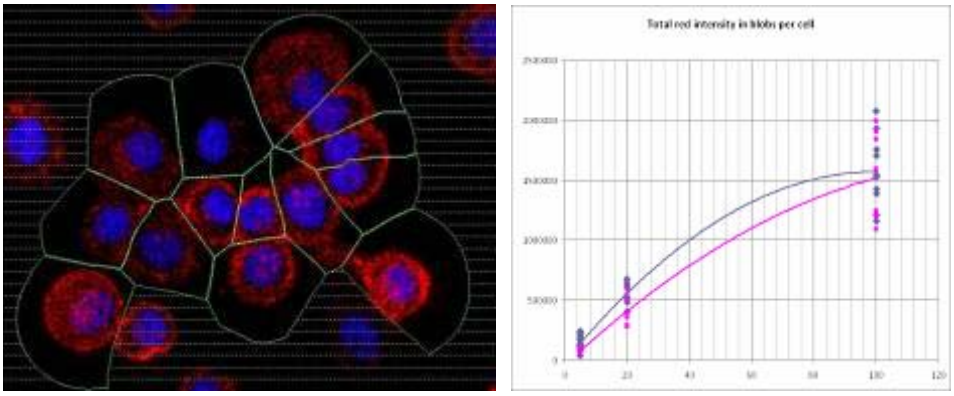

Figure 4. Cancer cells (blue nuclei) stained by the PLA technique for a characteristic biomarker (red signals): The Visiopharm software automatically segmented the individual cells and quantified the expression level of the biomarker.

VTT Technical Research Centre applied *in situ* PLA to study protein targets earlier identified in high-throughput screening on cancer cell lines using cell spot microarrays (CSMAs) and highly parallel RNAi screening to identify genes affecting cellular processes important in cancer progression. Using PLA, VTT could show which of the several possible integrin alpha-beta heterodimers in the cell that were activated/inactivated as a results of knockdown of other cellular genes by RNAi. For example, several genes that are required for the activation of the α5:β1 heterodimer in metastasizing prostate cancer cells were identified (Figure 5). These findings provide potential new cancer therapy targets, and the α5:β1 heterodimer specific PLA staining could find diagnostic use in the identification of aggressive tumour types.

Edinburgh University studied the role of specific heterodimers of the HER family receptors in tumours and their potential use as predictive biomarkers for successful chemotherapy of breast cancer. The PLA technology was successfully adapted to detect HER hetero- and homodimers in formalin fixed paraffin embedded (FFPE) tumour samples. Tissue microarrays from 322 women in the BR9601 trial, which compared cyclophosphamide, methotrexate, and fluorouracil (CMF) with epirubicin followed by CMF (epi-CMF), were analyzed for HER2 homodimers or HER2:HER3 heterodimers. Patients with high HER2 homodimer levels showed significantly decreased relapse free survival compared to low HER2 homodimer levels. Patients with high HER2:HER3 heterodimer levels showed significantly decreased relapse free survival (RFS) and overall survival (OS) rates compared to low HER2:HER3 heterodimer levels. There was also a trend that patients with high levels of HER2:HER3 heterodimers did not benefit from the addition of epirubicin to CMF. Conversely, patients with low levels of HER2:HER3 heterodimers showed increased RFS and OS rates when treated with epi-CMF compared with CMF.

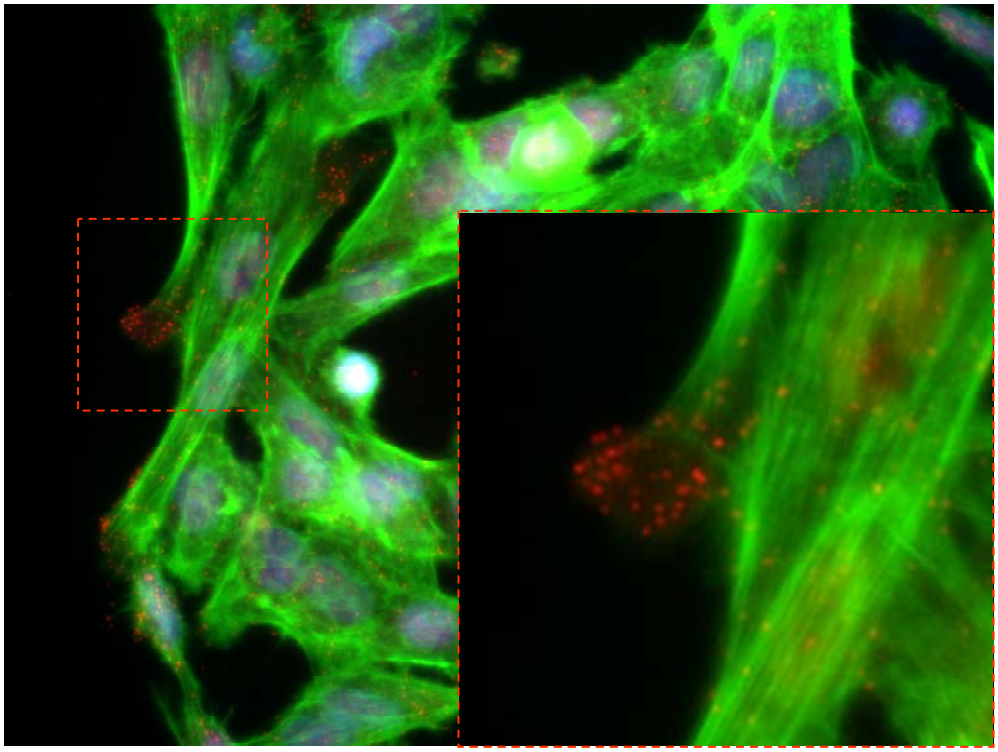

Figure 5. PLA staining of activated integrin α5:β1 dimers in a prostate cell line. The active dimers are localized in the cell's philopodia, the structures which the cells use for moving around. This staining pattern is a marker of motile cells, and can be used to study the invasion/metastasis potential of cancer cells, *e.g.* in a tumour.

Transcription factor AP-1 plays a critical role in the regulation of cell cycle progression and cell death, for instance upon exposure to specific hormones or DNA damaging agents. Leiden University Medical Centre (partner 8a) successfully applied the PLA technique to measure the interactions between the distinct components of transcription factor AP-1 (the various Jun, Fos and ATF protein family members), to be able to examine AP-1 composition and activity (including distinct post-translational modifications) in (a) human cells in culture - to test for instance the effect of anti-cancer drugs - and (2) in tissue preparations, including biopsies from cancer patients (Fig 6). Our results suggest that only a specific subset of AP-1 complexes can enhance breast cancer progression, by stimulating cancer cell migration. In addition, we have found that certain AP-1 complexes enhance glioblastoma cell death, whereas other AP-1 complexes rather trigger glioblastoma cell survival. These results indicate that AP-1 PLA is a promising diagnostic tool for cancer.

The padlock probe technology was furthermore used by Leiden University Medical Centre (partner 8b) to interrogate Mitochondrial DNA (mtDNA) nucleic acids and to distinguish closely similar sequence variants. mtDNA mutations play roles in cancer, mitochondrial diseases and aging. Because a cell contains 100s to 1000s of mtDNAs, expression of such mutation results only when the level of mutation reaches a given threshold (say ~700 of the ~1000 mtDNAs in a given cell). Mitotic segregation of inherited or acquired mtDNA mutations underlies mtDNA mutation accumulation, but the mechanism of mitotic segregation is elusive. LUMC successfully developed and applied mtDNA point mutation padlock probing and image analysis tools to study mtDNA segregation in long term cell culture, and found (contrary to generally accepted hypothesis of non-random segregation) patterns that could be related to genetic reorganization of the

nucleoid, the mitochondrial chromosome containing multiple mtDNAs. Additionally, a padlock probe method for *in situ* mtDNA deletion detection in skeletal muscle was successfully developed and applied (see Figure 7). The new technology enables research in causes of muscle wasting and weakness in elderly people.

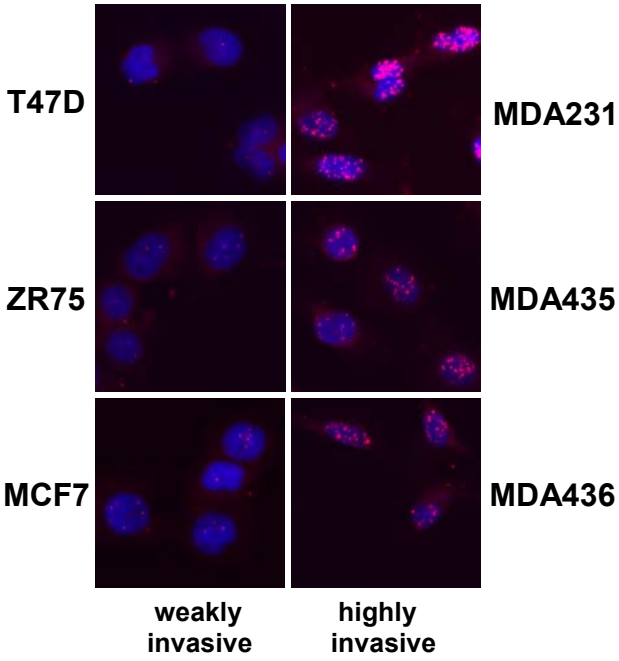

Figure 6. *In situ* PLA with antibodies against cJun and Fra1 for weakly invasive and highly invasive breast cancer cell lines. The interaction events are visible as red dots and nuclear staining in blue (DAPI).

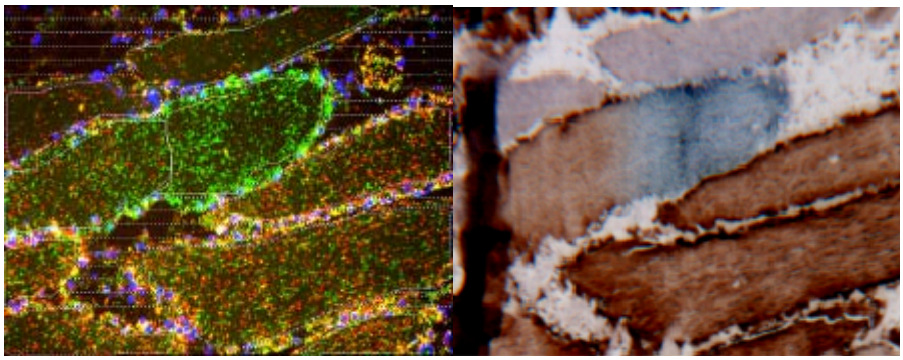

Figure 7. Padlock mtDNA deletion detection in skeletal muscle. Left: The intensely green area represents a segment of a muscle fiber with a very high percentage of mtDNA molecules with a functionally detrimental deletion. Areas with equal numbers of red and green signals indicates non-deleted mtDNA. Differences in the total number of signal signifies the different muscle types (oxidative, glycolytic). Right : A sequential section enzyme histochemically stained for cytochrome c oxidase (partly mtDNA coded; brown) and succinate dehycdrogenase (nuclear coded, purple) reveal corresponding segmental loss of cyt c oxidase in one of the oxidative muscle fibers.

Impact

ENLIGHT provided the scientific community with two new and unique molecular tools to study individual proteins or nucleic acids and their functional status in single cells and clinical specimens. This project consisted of three European SMEs (Olink AB, Visiopharm A/S, ImmunSystem AB) and one large established company (Dako Denmark A/S), all with high future potential. These companies were successfully brought together with academic scientists (Uppsala University, VTT Technical Research Centre in Finland, Edinburgh University, Leiden University Medical Centre). We expect that the *in situ* techniques developed in this project, and the scientific knowledge created, in the longer run will lead to improved disease prevention, more rapid and accurate cancer diagnosis, and better treatment opportunities. Relative to state-of-the-art procedures, these methods are expected to provide significantly improved *in situ* analyses in terms of specificity, sensitivity (single molecule detection), possibility to study biomarker localisation, analysis of protein interactions and protein modifications, and an opportunity for simultaneous analysis of multiple markers (multiplex analysis). Automated image analysis procedures were developed, i.e. software-based classification of molecules and their localisation in tissues or cells. The software provides a rapid way to analyse many samples as well as user-independent, unbiased data classification. The results from this project have provided important new commercial opportunities for products addressing significant market needs, thereby allowing the participating European companies to build sustainable businesses at the forefront of biotechnology.

Dissemination and use

The following exploitable results were achieved as a result of the project:

- Some aspects of the *in situ* PLA methods and reagents developed and explored within the ENLIGHT project was further developed into commercial products by Olink in a separate product development project. These products are now available from Olink AB under the brand name Duolink®. For information please contact CEO Björn Ekström at Olink AB.
- The image analysis tools developed within the project is available as freeware that can be downloaded from Uppsala University's home page: (http://www.cb.uu.se/~amin/BlobFinder). A new approach to quantification of colocalization and elimination of cross-talk in fluorescence microscopy images inspired by the research in the project was developed further in separate work and patented and is available for licensing, contact Ewert Bengtsson at Uppsala University for information. Certain of the algorithms developed within the project were converted and verified in a separate project and are now integrated as new functions in the commercially available image analysis package "VIS" from Visiopharm. For information please contact Niels Foged, CSO at Visiopharm.
- The antibodies developed within the project were specified by the participants in WP3 to aid in the studies undertaken in the project. These antibodies will become commercially available in the near future from ImmunSystem AB after further qualification and product development. For more information contact Anders Larsson at ImmunSystem AB, or go to www.immunsystem.com.