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Intermediate report on tools and methodologies for the study, diagnosis and therapy of human disease

Lead Beneficiary: 13 LENS

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Deliverable Nature	
R = Report, P = Prototype, D = Demonstrator, O = Other	R
Dissemination Level	
PU = Public PP = Restricted to other programme participants (incl. the Commission Services) RE = Restricted to a group specified by the consortium (incl. the Commission Services) CO = Confidential, only for members of the consortium (incl. the Commission Services)	PU

A. Abstract / Executive Summary

During the first 24 months of the project, LENS has been working on the development of different optical and microscopy techniques for the study, diagnosis and therapy of human diseases.

LENS exploited a combination of non-linear microscopy techniques (TPE, SHG, FLIM, SLIM) and developed methodologies for the diagnosis of skin cancer.

LENS explored basic features and anomalies in the spatiotemporal relationship between intracellular Ca^{2+} fluxes and action potential propagation, the concurrent determinants of cellular remodelling in cardiac diseases. This investigation was made possible thanks to the development of a new optical approach capable of probing cardiac samples in random access modalities.

Moreover, LENS systematically applied single molecule tracking techniques to investigate the molecular basis of Alzheimer's disease. This approach has allowed discovering new features normally not accessible with standard methods based on data averaging.

B. Deliverable Report

1 Introduction

Skin cancer: Non-linear optical (NLO) microscopy offers promising solutions for both in vivo and ex vivo tissue imaging at sub-cellular level and it can provide both morphological and functional information in a label-free modality. The combination of NLO microscopy techniques providing morphological information, together with those providing functional information is a crucial issue for tissue classification and pathological assessment.

Cardiac pathologies: The heart is constituted of excitable and contractile cells, called cardiomyocytes. Intracellular Ca^{2+} fluxes mediate the transduction between the electrical activity of cardiomyocyte membrane (sarcolemma) and the mechanical function of the contractile units (sarcomeres). Action potential (AP), via the transverse axial tubular system (TATS), synchronously triggers uniform Ca^{2+} release from the sarcoplasmic reticulum (SR) throughout the cardiomyocytes. This process is known as excitation-contraction coupling (ECC). ECC abnormalities lead to severe arrhythmias and contractile dysfunction, the two main features of human cardiac diseases. Although multifaceted, a common trait of these abnormalities consists in the loss of the spatiotemporal relationship between membrane voltage and Ca^{2+} fluxes. Why and how this occurs has been studied for decades, but remains a matter of debate due to the lack of tools and techniques able to *shed light* on ECC microenvironment.

Alzheimer's disease: Most of the current research on the molecular mechanisms of Alzheimer's disease is based on averaged results obtained using bulk methods. In this case, many important details can be missed and only the most prominent features are eventually taken into account. This consideration may explain at least part of the discrepancies arising from the several models that have been proposed during the last years. Within this context, we aimed at providing a better understanding of the pathogenesis of Alzheimer's disease by studying the dynamic features of this complex system at a single molecule level.

2 Objectives

Skin Cancer: The designed study aimed at using multimodal multiphoton microscopy and spectroscopy for in vivo early diagnosis of skin cancer.

Cardiac pathologies: This subproject is devoted to explore basic features and anomalies in the spatiotemporal relationship between intracellular Ca^{2+} fluxes and AP propagation, the

concurrent determinants of cellular remodelling in cardiac diseases. This investigation will be made possible thanks to the development of a new optical approach capable of probing cardiac samples in random access modalities. Ultrafast deflectors will be used to rapidly scan laser beams across the sample performing multiplexed optical measurements of membrane potential and intracellular Ca^{2+} across cell and tissue. The spatiotemporal relationship between Ca^{2+} and voltage will be dissected in a well-defined pathological model of heart failure.

Alzheimer's disease: The goal of this part of the deliverable was to provide a better understanding of the pathogenesis of Alzheimer's disease by studying the dynamic features of this complex system at a single molecule level. It consisted of three main objectives:

1. Monitor the surface mobility of single A-beta oligomers in living cells.
2. Challenge the controversial hypothesis that supports the specific binding of A-beta oligomers to lipid rafts.
3. Compare the membrane dynamics of different amyloid oligomers.

3 Work performed / results / description

Skin cancer In vivo imaging

During the period we are reporting about, we built a custom multimodal multiphoton microscope inside a research lab located in the dermatological clinic of the University of Florence (Azienda Ospedaliera Firenze Centro – c/o Villa Santa Chiara). In addition, we prepared the Clinical Protocol to be submitted to the Local Ethical Committee for in vivo studies.

Unfortunately, due to the regional spending review policy on health assistance, related to the current economic crisis, the Dermatological Clinic has moved in another building and the spaces dedicated to the clinical dermatological activities have been drastically reduced, making impossible to have a research lab dedicated to clinical investigation with new optical devices in the new location. For this reason, the developed devices have been moved to Lens, limiting the research activity to be carried out to ex vivo tissues.

Our new plan is to perform research activity on ex vivo fixed tissue slices of various biological tissues (not only skin), instead of in vivo on living subject, using the same optical methods. The activity could be turned again to in vivo investigation in the future, provided that a proper location for the experimental setup is found within the dermatological hospital.

Skin cancer Ex vivo imaging

During the period we are reporting about, we performed imaging of various ex vivo tissue samples using multimodal non-linear microscopy. In particular, we focused our attention on both cancerous and non-cancerous (atherosclerosis) diseases using both thin slices and fresh massive biopsies of tissues.

For cancerous lesions, samples of healthy colon mucosa were examined and compared to both adenomatous and adenocarcinoma tissues. Images acquired using TPF microscopy on fresh colon biopsies demonstrated good correlation with conventional histological examination carried out on the same samples. A quantitative morphological analysis classified tissues on the basis of both nucleus-to-cytoplasm ratio and cellular asymmetry. Functional characterization, performed by comparing the relative intensities of NADH and FAD fluorescence, demonstrated an altered metabolic activity in both adenomatous polyp and adenocarcinoma compared to healthy mucosa. In particular, adenomatous tissue showed a metabolic activity more similar to adenocarcinoma than to healthy mucosa.

For non-cancerous lesions, we demonstrated that SHG microscopy can be used to image both cholesterol and collagen and to discriminate the two molecules by means of a detailed

analysis of the detected SHG signal. In particular, we first provided a demonstration of the potential of SHG for imaging cholesterol deposition within atherosclerotic plaques. Then we focus the attention on forward and backward scattered SHG microscopy, demonstrating that the simultaneous detection of forward and backward scattered SHG signal can be successfully used for discriminating cholesterol deposition from connective tissue in the arterial wall. Finally, image pattern analysis methods were implemented and used for scoring collagen organization in plaques against normal arterial wall, finding altered collagen architecture within plaques. The used methods allowed quantitatively determining collagen fibres anisotropy, size, and spacing in both atherosclerotic depositions and normal arterial wall.

Random Access Multiphoton Microscopy of Cardiac pathologies

Here, we develop an imaging method to simultaneously assess TATS electrical activity and local Ca^{2+} release. We found that, in hear failure cardiomyocytes, sites where TATS elements fail to conduct AP show a slower and reduced local Ca^{2+} transient compared to regions with electrically coupled elements. Moreover, spontaneous depolarisation events occurring in failing TATS elements can trigger local Ca^{2+} release, resulting in Ca^{2+} sparks. TATS electrical remodelling is a major determinant of altered kinetics, amplitude and homogeneity of Ca^{2+} release in heart failure. These abnormalities, together with the occurrence of tubule-driven depolarizations and Ca^{2+} sparks, may contribute to the arrhythmic burden and strongly suggest a central role of TATS remodelling in heart failure.

Single molecule imaging of Alzheimer's disease

The work has been mainly based on single particle tracking methodologies. These involve the labeling of the molecule of interest with specific fluorescent probes in living cells, imaging the dynamic behavior of single molecules with highly sensitive detectors, and analyzing recorded movies with custom-made algorithms to extrapolate data on diffusion. A custom TIRF fluorescent microscope has also been developed during this deliverable.

By taking advantage of this approach, we have successfully addressed most of the objectives described above:

1. By using specific antibodies coupled to highly fluorescent probes, we have been able to track single Abeta oligomers on the plasma membrane of neuroblastoma cells, and found that their mobility is limited.

2. Simultaneous imaging of Abeta oligomers and lipid rafts labelled with cholera toxin (it binds specifically to GM1, a ganglioside characteristic of lipid rafts) has shown that Abeta oligomers can interact with and decrease the mobility of the raft components.

3. Single particle tracking of amyloid aggregates composed by amylin or sup35 prion display a confined mobility on the plasma membrane, comparable to the structurally similar Abeta oligomers. These aggregates were also found to reduce the mobility of GM1.

We have also collaborated to a study on lipid rafts mobility during cell polarization, and to another one on the interaction between the LDL receptor and IDOL, a protein involved in its degradation. These studies are related to the development of cancer and atherosclerosis, respectively.

4 Conclusions

The morpho-functional analysis carried out on ex vivo tissues using multimodal non-linear microscopy may represent a powerful tool for early diagnosis of tumors and may also be extended to the diagnostics of other tissues. In addition, the methods represent a support, or also a competitive label-free diagnostic alternative to standard histopathological methods for evaluating biological tissues. Further, the methods developed could be translated to in vivo diagnostics, once the in vivo imaging facility will be restored.

Our results show that functional defects of TATS elements occurring in heart failure, significantly contribute to the pathophysiology of this disease and needs to be addressed by future therapeutic strategies aiming to reduce contractile dysfunction and arrhythmias in patients.

The study on Alzheimer's disease has contributed to add new information on the biology of the toxic Abeta petide oligomers by describing their dynamic behaviour on the plasma membrane of living cells. Additional results on the interaction of Abeta oligomers with components of the plasma membrane have also provided proof of evidence for a mechanism of toxicity that was object of debate in the past. Although the interaction of Abeta oligomers with the cellular membrane is a well-known process, the manner through which they cause cell dysfunction can depend on several factors. The findings related to this deliverable have revealed a new potential mechanism of toxicity attributable to the loss of function of specific membrane components consequent to an alteration of their mobility caused by the binding to slowly diffusing Abeta oligomers. The dynamic behaviour and the membrane effects of toxic amyloid aggregates formed by proteins or peptides other than Abeta have also been object of investigation. Results similar to those obtained in the case of Abeta oligomers were found for amylin (involved in the development of type II diabetes) and prion sup35, supporting the hypothesis that amyloid diseases share similar mechanisms of toxicity.

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