Photonic System for Liquid Biopsy - PHIL

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ABSTRACT

Liquid biopsy (LB) represents an important tool for modern oncology that enables increasingly safe, personalized, and robust cancer diagnosis and treatment. State-of-the-art LB systems require the amplification of the DNA in the blood samples, a cost and time-consuming process. Using low light level photosensors and arrival timing information, we aim with PHIL to avoid the amplification stage which would be a breakthrough in the field of molecular diagnostic in oncology. In this article, we summarize our achievements during the project and our future vision.

Keywords: liquid biopsy, oncology, photon detection system, integration

1. INTRODUCTION

Many strategies to analyse plasma tumor DNA (ptDNA) in clinical practice and clinical development have emerged during the last years:

(i) Digital droplet polymerase chain reaction PCR (ddPCR) increases analytical sensitivity and enables the detection of one mutant DNA molecule per 100.000 normal DNA molecules with low signal-to-noise ratio.

(ii) Tagged amplicon deep sequencing (TAm-Seq) uses targeted amplification followed by next-generation sequencing enabling the sequencing of entire genes of interest as well as scrutinized for genetic alterations without a priori knowledge of a tumour's driver mutations.

(iii) Cancer personalized profiling by deep sequencing (CAPP-Seq) can detect one molecule of mutant DNA in 10,000 molecules of healthy DNA and lead to the identification of mutations of genomic DNA in more than 95% of the tumours.

(iv) Silico–based integrated digital error suppression (iDES) technique has been developed to overcome the limitation of low quantities of ptDNA in the blood and sequencing artefacts currently limit analytical sensitivity and,

(v) Targeted error-correction sequencing (the TEC-Seq) is ultrasensitive direct evaluation of sequence changes in circulating cell-free DNA using massively parallel sequencing. Current state-of-the art techniques do require a complex workflow, costly equipment and reagents, waste generation and slow read out times. Now, no direct detection system is available, without DNA amplification and minimum sample processing, has been developed so far.

PHIL project aims to reach the proof of concept of the direct DNA mutation detection without amplification of the EGFR gene, one of the most commonly mutated in non-small cell lung cancer but also mutated in most common cancers such as colon, breast and prostate among others. By using specific mutation molecular beacons combined with ultrasensitive photonic platform, it will be possible detect directly circulating cancerous mutations of genomic DNA, turning out into a breakthrough in molecular diagnostics in Oncology and extendable to other diseases

During the project, the prototype of the PHIL platform was successfully designed, assembled and commissioned. The reference measurements with the developed molecular beacon were performed using a commercial TECAN Infinite MPlex. Preliminary results using DNA and molecular beacon samples were also carried. The setup will be used in the next months for further studies for cancer and COVID-19 applications.

2. STATE OF THE ART

Liquid biopsy is an analytical test for the diagnosis and monitoring of cancer disease. Then tumors are in metastatic phase, they release cells and, therefore, genetic materials (tumor DNA, also called ptDNA) in the bloodstream. So, it is possible to detect ptDNA with a simple blood test [1]. However, these are found in very small quantities

(~picograms/microliter) and are very difficult to detect. The detection of ptDNA is expensive and time consuming (more than 2 hours). Many strategies to analyse ptDNA in clinical practice and clinical development have emerged during the last years [2]: (i) Digital droplet polymerase chain reaction PCR (ddPCR) increases analytical sensitivity and enables the detection of one mutant DNA molecule per 100.000 normal DNA molecules with low signal-to-noise ratio. (ii) Tagged amplicon deep sequencing (TAm-Seq) uses targeted amplification followed by next-generation sequencing enabling the sequencing of entire genes of interest as well as scrutinized for genetic alterations without a priori knowledge of a tumour's driver mutations. (iii) Cancer personalized profiling by deep sequencing (CAPP-Seq) can sequence one mutant DNA consist of 10.000 molecules and lead to the identification of mutations of genomic DNA in more than 95% of the tumours. (iv) Silico–based integrated digital error suppression (iDES) technique has been developed to reduce background noise signal significantly increasing the sensitivity, specificity, and accuracy for ptDNA in the blood and , (v) Targeted error-correction sequencing (the TEC-Seq) is ultrasensitive direct evaluation of sequence changes in circulating cell-free DNA using massively parallel sequencing. The adoption of these techniques has been slowed down due to a complex workflow, pre-treatment of the sample, costly equipment and reagents, waste generation and slow read out times (several hours). Now, no direct detection system is available, without DNA amplification and minimum sample processing, has been developed so far.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

Current state-of-the art techniques do require a complex workflow, costly equipment and reagents, waste generation and slow read out times. Now, no direct detection system is available, without DNA amplification and minimum sample processing, has been developed so far.

PHIL project aims to reach the proof of concept of the direct DNA mutation detection without amplification of the EGFR gene, one of the most commonly mutated in non-small cell lung cancer but also mutated in most common cancers such as colon, breast and prostate among others. And, EFGR is targeted by a number of marketed cancer therapies. By using specific mutation molecular beacons combined with ultrasensitive photonic platform, it will be possible detect directly circulating cancerous mutations of genomic DNA, therefore providing key information to practitioners to decide on the disease progression, therapy selection and patient prognosis. PHIL turns out into a breakthrough in the field of molecular diagnostic in Oncology.

4. PROJECT RESULTS

In this section, first the prototype designed and built during the project will be presented. The setup was characterized and optimized. Then, some reference measurements are presented before preliminary results with DNA samples and molecular beacon are presented. Due to the COVID-19 situation which for 4 months did not allow to access the laboratory and also afterwards slowed down the progress due to the implemented restrictions, these results are only preliminary and the developed platform will be used in the next months for further tests for cancer and COVID-19 detection including an optimization of the analysis. Special focus will be laid on the timing analysis.

PHIL Prototype

A photo of the core elements of the setup is shown in Fig. 1. A nanosecond long laser pulse is directed on the sample. Depending on the amount of target DNA the95light will be shifted to longer wavelengths. Photosensors are placed perpendicular to the laser beam to detect these photons. To reduce the background coming from stray light of the original laser light, optical long pass filters are placed between sample and photosensors.

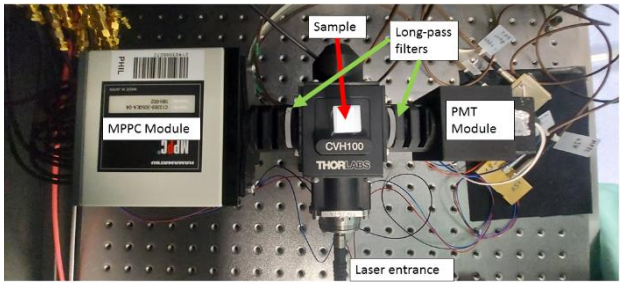

Fig. 1. Photo of the prototype setup: The MPPC and the PMT module are photosensors.

For the laser, the NPL49B from ThorLabs was chosen with a selected pulse width of 10 ns. The wavelength of the device was measured to be 490 +/- 2 nm, close to the maximum of the absorption peak of the molecular beacon.

For the photon detection two different photosensors were selected: a MPPC module (Hamamatsu: C13369-1173050EA-04) consisting of an array of 4x4 MPPCs each of 3x3 mm2. This sensor array allows to detect easily single photons. The second photosensor is PMT module (Hamamatsu: H13543-300) with a gain of 10^6 allowing to detect around 10 and more photons.

The signals are recorded with a DT57432 digitizer from CAEN. This ADC provides a sampling of 5 Gs/s and a maximal acquisition window of about 200 ns. The high sampling rate makes this device ideal of timing applications. High performance optical filters from Edmunds were acquired and placed between the sample, a 0.1 ml cuvette, and the photosensors. The light guiding structure and sensor supports were designed in-house and produced by 3D printing.

All components were first characterized before the integration and the geometry was optimized to increase the signal to background light ratio.

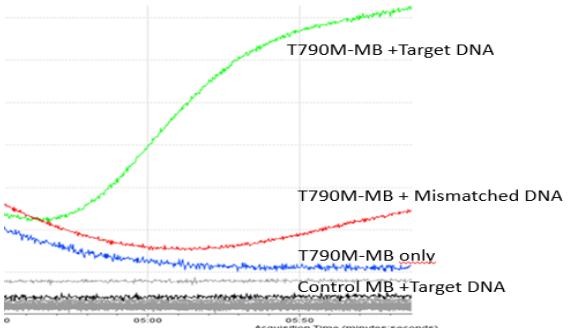

Fig. 2. Green curve demonstrates the light of 6-FAM produced by molecular beacon bind to tumour DNA. Red curve demonstrates the light of 6-FAM produced by molecular beacon bind to mismatched DNA. It is demonstrated that it is specific only for target DNA.

Reference measurements

For detection the specific mutation of T790M EGFR exon molecular beacons was designed. Molecular beacons are hairpin-shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid sequence. Tumour liberated mutated T790M EGFR gene DNA binds specifically to this molecular beacon with an internally quenched fluorophore and open this probe to restore fluorescence and give the signal. (Fig. 2).

Preliminary results with the PHIL prototype

For the first measurements, the detection of different concentration of Target DNA with T790M molecular beacon in binding buffer is studied. The preliminary results are shown in Fig. 3. For this study, different filters were used for both photosensors. Even for the smallest concentration of 0.0001 uM one can observe a difference of about 10% more light yield compared to the case that no target DNA is presence.

For this first study, only the light yield is exploited. The timing information, which in the previous studies gave promising results will be used in further analysis of the data.

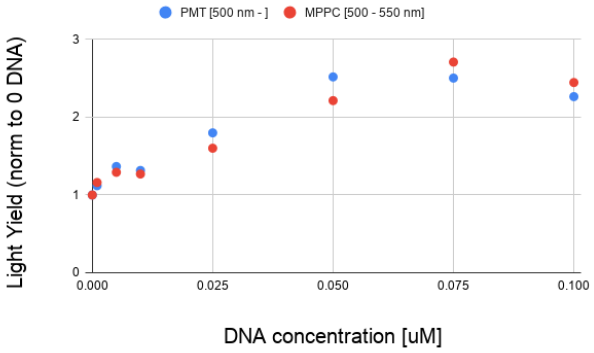

Fig. 3. The light yield in arbitrary units in function of the DNA concentration for PMT (blue) and MPPC (red) modules. For the PMT only a 500 nm long-pass filter was used, while for the MPPC an addition short-pass filter of 550 nm was placed between sample and MPPC.

An effective way to increase the sensitivity of the system was found during the tests. It turned out that an important limitation of the system in the case of very low light levels is coming from noise induced in the system by the external trigger as shown in Fig. 4. As mentioned in the next section we consider this option for a prototype for a cost-effective device.

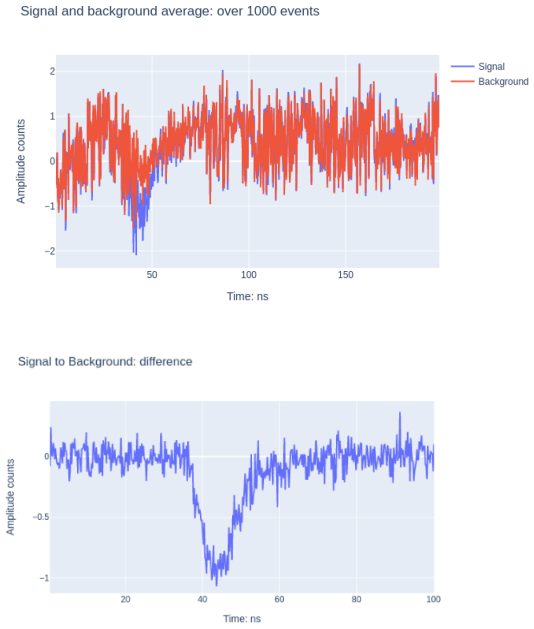

Fig. 4. (Top) Signal and background overlaid in the same plot (average over 1000 events). (Bottom) Subtracting one from the other eliminates the noise pattern induced by the trigger cleaning up significantly the signal.

5. FUTURE PROJECT VISION

As mentioned before, the results are still preliminary but nevertheless promising and based on these we have started to make develop a vision for the future. This includes: ongoing patent application, identification of key partners for industrial scale up and market entry, already started system tuning to the detection of COVID19 in saliva, and develop the concept of a DNA/RNA biofluid analysis platform.

5.1. Technology Scaling

At the end of the project, we have in our hands a working prototype which allows to detect light levels down to single photons with precise arrival time information. Depending on the final results, we consider generating a technology pipeline containing 2 approaches for the technology scaling:

No timing

A very cost-effective concept and easily to be scaled to TRL 6-7 could exploit the approach shown in Fig. 4, subtracting the background from the signal eliminating noise patterns. During the project, an electronics design implementing this concept on the analogue signal level was

already developed and the PCBs produced. No high sampling ADC is needed for this, which significantly reduces the costs. Tests are ongoing.

Timing

In the case that it turns out, that the timing information provides significant better performance, more steps will be needed:

a. For the current project, commercial electronics is used for the digitizing and storage of the data. Commercial Gs/s ADC chips are available but the optimized electronics and fast DAQ needs to be developed to increase the data taking rate to at least 1 kHz while reducing the costs to reasonable levels for commercial applications. This integration work would also require including an OEM laser module.
b. Tests with additional molecular beacons which might emit at different wavelengths should be foreseen to open the possibility of multiplex analyses. The filter system would need to be developed accordingly.
c. Clinical tests at a hospital to develop the system in an optimal way for real applications. Leitat and IFAE have both contacts with the hospital Parc Tauli in Sabadell (Spain).
d. Desirable, but not mandatory, would be to investigate with a chemistry institute if the decay time of the molecular beacon could be increased to several nanoseconds to improve the sensitivity of the system.

5.2. Project Synergies and Outreach

At the of the phase 1, PHIL project will deliver a fully functional prototype validated with controlled samples of mutated EGFR DNA and coronavirus, ready to progress from TRL 4/5 up to the market. A second project phase planning is already defined. It is structured into 5 main activities: (1) Industrial scale-up, (2) IPR protection, (3) Regulatory compliance & market entry, (4) Clinical validation and, (5) public & private funding. Key partners have been already identified for each of the main activities foreseen:

a. CMOs complying ISO 13485 standard and CE marking for device and microfluidic cartridge development, validation & manufacturing,
b. IPR and regulatory affairs advisors,
c. Tertiary hospitals for clinical trial including KOL and technology prescribers,
d. Patient associations

PHIL is committed to the timely and effective dissemination. The immediate or future potential end users are varied and expected to in the dissemination scope: the scientific community, SMEs, Pharma, MedTech/Biotech Start-ups, Medical practitioners, government institutions Healthcare Providers, Patients & their families, Decision Makers, Policy makers and Patient Associations. The dissemination Plan is aimed at divulging the main innovative aspects which evolve during the development of the project in accordance with IPR restrictions. This will give clear measures and priorities of how to spread the solutions elaborated by the project consortium to the different stakeholders: industry, biomedical society, and general audience. The main dissemination activities that are foreseen in the project are outlined below:

- A project web portal will be established for the exchange of publicly available information about project and consortium partners.
- Results from the project will be reported at major relevant scientific conferences
- Promotional material in the form of on-line pages, brochures, posters, videos and similar will be prepared
- Results from the project will be published in related scientific journals that are market relevant. This will primarily include publication of mature results containing in-depth methodical analysis and discussion Such dissemination activities will be carried out through publications in peer-reviewed journals (Open Access)
- The dissemination of the experience and know-how among the specialists will be pursued through a dedicated workshop on project topics
- General press and through the communication channels available through the patient's association

5.3. Technology application and demonstration cases

The demonstration case for PHIL technology is focused on health in its broad sense. To **improve clinical decisions and health outcomes of cancer patients,** more particularly lung cancer patients: tracking tumor evolution and adaptation over time by mutation analysis. Then, the quality of prognosis is improved plus the selection of the most effective therapy for each genetic profile according to the individual mutation profile obtained. And , the **Improvement of social and healthcare system**, the technology will enable: (a) To increase the number of patients which are currently under-screened. E.g. 20% of stage IIIb and IV non-small cell lung cancer patients have no access to testing due to a lack of or inadequate tissue biopsy (b) To serve as a "liquid re-biopsy" for monitoring and prognosis in: biomarker models, where there is correlation of active mutations and poor prognosis; response to treatment, when the tumor evolves to become resistant to treatment regimen; or stratification to identify subsets of patients, and, (c) To allow targeted therapies based on specific gene(s) mutations (e.g. EGFR, BRAF, PI3K,etc.) based on the patients' particular disease profile. So, avoid unnecessary therapies that might be non-effective but due to severe side effects and extremely strainful for the patient. Thus, quality of life of a large group of patients can be improved.

Meanwhile, the current COVID-19 pandemic came up. This challenging situation and the extraordinary diagnostic need,

it was decided to adapt the prototype developed to detect coronavirus in saliva samples as we did with EGFR mutation. It has been designed specific markers to detect the presence of the virus and satisfactory detection tests have been carried out with the available prototype. The PHIL technology developed overcomes the PCR barriers (time-to-results, supply chain, sample management) giving a rapid response and decentralizing the detection of the coronavirus

The high sensitivity and the reduced time-to-results of the PHIL's technology make it a great building block for a DNA/RNA biofluid technology platform for high throughput diagnostics which can turnout into pre-existing ERICs.

5.4. Technology commercialization

The commercialization roadmap is already envisaged, a patent application is being prepared as important previous step to transfer the technology into a start-up which will apply for public and private funding to lead the PHIL technology development and bring it into the molecular diagnostic market. IFAE and Leitat have proven experience in tech-transfer, spin-out creation and fund raising. Moreover, key MedTech players concentrate in the Barcelona area: academia, business schools, industrial partners, innovation centres, 50 international investors + nationals, VC offices. The business model is based on a technology-platform model which will deliver devices and specific reagents to an expandable number of diseases where nucleic acid detection is relevant, and a market need exists.

5.5. Envisioned risks

As a general framework, the ISO 14971:2019

Medical devices (Application of risk management to medical devices) will be considered. Beside this framework some putative risks have been identified: **Technical**, issues on background light extraction coming from biofluid (serum, plasma, saliva). We have found this issue that is under study by filtering the specific wavelength coming from the molecular beacon. **Regulatory,** it is known that the EU regulation on medical devices and in vitro diagnostics is under review thus special monitoring will be performed to adapt the project plan accordingly. **IPR**, the patent strategy is to create a strong patent family protecting different elements of the diagnostic device. **Analytical performance**, attention will be set on negative predictive value, positive predictive value, sensitivity, and specificity. If any of the above do not comply the requirements defined according to the device intended use, new molecular beacon and/or fluorescent probe, shift on wavelength, and/or photonic design will be reviewed to reach

5.6. Liaison with Student Teams and Socio-Economic Study

Presently, a MSc student is participating in the PHIL project under the supervision of Dr. Lux. For the next, phase at least 2 students will be joining the project. IFAE as institution part of the Autonomous University of Barcelona, regularly hosts PhD and MSc students. On the other hand, Leitat has collaboration agreement with many Spanish and non-Spanish universities, participates in Marie Skłodowska-Curie Actions, TecnioSpring and others young research fellowships as part of its regular research activities.

Previous experience in organizing summer schools for pre-graduate and post-graduate students together with KTH and GE-Healthcare (Hungary) on Healthcare & Artificial intelligence funded by EIT-Health (HelloAI-2018, HelloAI-2019 & HelloAIRIS-2020 totally virtual) grants to the current consortium the tools, resources and experience in organizing international training either way, in person or remotely, complex subjects. For the phase 2, it's planned to setup a Moodle platform providing the contents and activities to facilitate design thinking and project development to provide ideas and prototypes inspired by the projects technology for addressing Societal Challenges. Current members of the consortium will be involved both from the physics side (Dr. Lux) and from the biology side (Dr. Grynite, Dr. Cabot)

For the expert-driven socio-economic study, both institution hold a broad and international academic and industry network; particularly Leitat which is currently participating in 63 H2020 projects and it's member of several technological platforms, namely: EPOSS, ETPN, Photonics21, EIT-Health, EARTO, WAITRO among others. All these scientific and industrial fora will be at ATTRACT disposal for the study.

6. ACKNOWLEDGEMENT

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7. REFERENCES

[1] M. D. Kessler, N. R. Pawar, S. S. Martin, T. M. Antalis, T. D.253O'Connor,Improving cancer detection and treatment with liq-254uid biopsies and ptdna, Trends in Cancer 4 (2018) 643 – 654.

[2] X. Han, J. Wang, Y. Sun, Circulating tumor dna as biomarkers for258cancer detection, Genomics, Proteomics and Bioinformatics 15 (2017)25959–72. doi:10.1016/j.gpb.2016.12.00