

# Team Results Document

## TwentUs



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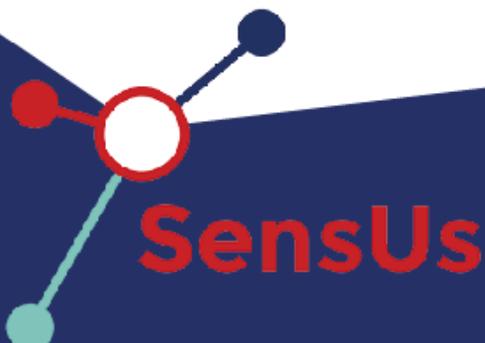
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**11.08.2023**



SensUs 2023  
Traumatic Brain Injury

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## Abstract

We present a biosensor designed for sports applications, aiming to improve traumatic brain injury (TBI) assessment in rugby and American football players. Leveraging electrochemical impedance spectroscopy (EIS) and selective surface coating, with our sensor we provide early and objective diagnostics to help athletes get the best recovery possible.

Our report demonstrates the foundational principles driving our assay and detection system. In our biosensor we use antibodies (Ab) for precise molecular recognition of alfa-Glial Fibrillary Acidic Protein (GFAP- $\alpha$ ). The complex formation of GFAP- $\alpha$  with the anti-GFAP Ab is detected via EIS and quantified.

We delve into cartridge technology, showcasing a disposable microfluidic device simplifying sample treatment. The user-instrument interface encompasses tailored hardware, software, and an intuitive user-interface. The embedded electronics approach allows for on-site data analysis.

The Translation Potential section introduces a comprehensive business model, highlighting the need for a fast-testing solution of TBI diagnosis in sports, especially ones with high physical contact. A business strategy is introduced outlining the development stages and key activities. Starting our business in the research market in Italian rugby before expanding to the European commercial market and working towards the American Football clubs in the NFL.

## Biosensor system and assay

### Molecular recognition and assay reagents

GFAP- $\alpha$  is the targeted analyte for the biosensor. Antibodies offer a highly specific interaction to form of selective coupling interaction in biological systems and thus, this concept was exploited in our design. For our experiments we used human IgG1 monoclonal antibodies produced in mice, see Appendix I.

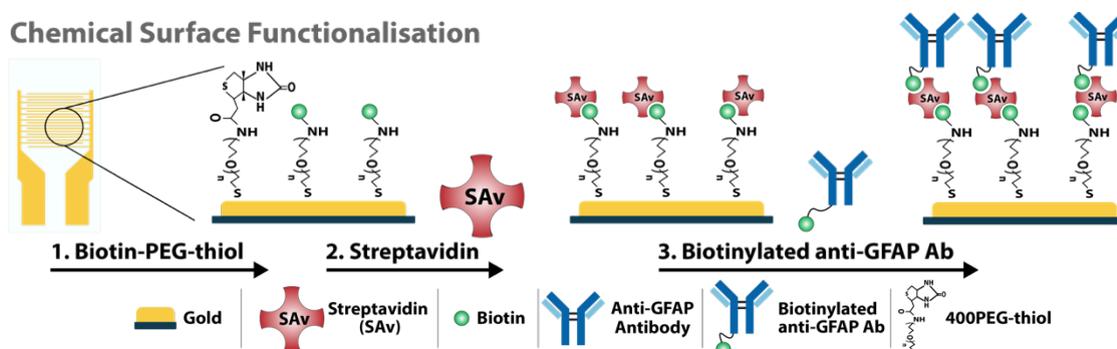


Figure 1: Schematic representation of the electrodes chemical surface functionalisation.

The electrode surface is coated via a multi-step protocol, illustrated in Figure 1. The initial step is gold sputter deposition onto glass chips, followed by subsequent functionalization for anti-GFAP Ab attachment. Our methodology consists of a Biotin-400PEG-thiol layer, serving as a protein interaction point and metal surface passivation layer. To counter non-specific binding on the gold surface, we applied 6-Mercapto-1-hexano (MCH) serves as a backfilling agent.

Streptavidin (SAv)-biotin bond is one of the strongest noncovalent biological interactions with a dissociation constant of of  $1 \times 10^{-14}$  M (Hamming & Huskens, 2021). Therefore, SAv-Biotin chemistry is widely used to build multi-layered coatings. SAv is a tetrameric molecule that is capable of binding four biotinylated molecules (Dubacheva et al., 2017), as such it is able to connect with the biotin of the thiol layers and, orthogonally with a biotinylated antibody. To introduce biotin functionalization on the anti-GFAP Ab, while preserving their functionality, oYo-Linkers, photoactivated at 365 nm, covalently attach biotin to the anti-GFAP Ab's heavy chain.

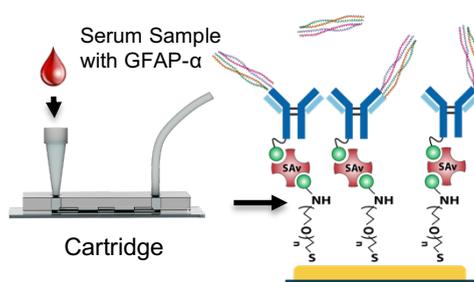


Figure 2: GFAP- $\alpha$  serum triggers essential antibody binding, a key molecular recognition event.

The resulting biotinylated antibody interacts with the SAv molecule, forming a crucial component of our system. To minimise non-specific binding of serum proteins during the measurement, the sensor surface is pre-treated with the same serum after the anti-GFAP Ab functionalization. This systematic approach reduces available binding sites on the bioreceptor layer, minimizing the influence of the non-specific bindings. This helps prevent issues with different types of patient plasma, which can vary and cause less predictable binding to unrelated substances. The final step is depicted in Figure 2. To ensure bulk uniformity, measurements follow phosphate buffered saline (PBS) – Plasma – PBS sequence, maintaining a consistent PBS environment. This mitigates plasma variability, aligning with the criterion for accurate, reliable measurements. In all the steps, a flow rate of 60  $\mu\text{L}/\text{min}$  is set to ensure ample diffusion area across the electrodes (Squires et al., 2008).

### Physical transduction

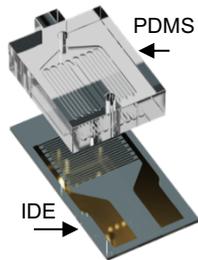
Analyte binding to antibodies alters the electrode-buffer interface dielectric properties as it traverses the biosensor surface, impacting impedance, detectable through EIS which is further explained in Appendix II. EIS meets necessary detection limits for analytes akin to GFAP- $\alpha$ 's weight, quantifying GFAP-antibody complex formation.

During the measurement process, a solution ferrocene is mixed with PBS to act as a redox mediator, facilitating the generation of a current that permeates the surface layers. As the GFAP-antibody complex forms, an additional layer is introduced to the surface, resulting in a detectable change in the concentration of GFAP- $\alpha$ . This alteration in concentration is discernible through analysis, providing valuable insights into the molecular interactions taking place on the biosensor's surface.



Figure 3: Gold-plated planar IDE

Interdigitated electrode (IDE) chip architecture was chosen for an increased electrode-buffer interface volume, leading to signal amplification. With the use of a COMSOL simulation, a grooved non-planar IDE chip has been designed and geometrically optimized for sensitivity. The fabrication method is detailed in Appendix III. Despite promising initial attempts at implementing the complex chip design, challenges in terms of time and cost prompted a strategic shift towards a transition to simplified planar variant (see Figure 3).



*Figure 4: Rendered design view. The PDMS channels can be seen over the interdigitated electrode layer.*

### Cartridge technology

The TwentUs biosensing device functions by using a micro-pump to maintain a precise flowrate of sample through the chip/cartridge channels during a test. The chip consists of a Polydimethylsiloxane (PDMS) layer (with 300  $\mu\text{m}$  wide, 100  $\mu\text{m}$  high microfluidic flow channels) positioned over the top of interdigitated gold electrodes, as shown in Figure 4. The assembly alignment is enhanced using a 3D-printed chip hold, which aids the binding of the IDE and the PDMS surfaces layers by acting as a guide.

Fully functional cartridges can be inserted into the device, where a clip-on electrode design makes it easy to (dis)connect them. The TwentUs cartridges can also be partially refurbished and reused, specifically the IDE can be cleaned and washed in Piranha solution. This is also the costliest part of a chip. It makes financial and environmental sense to reuse it, and we base part of our business plan on it.

### Reader instrument and user interaction

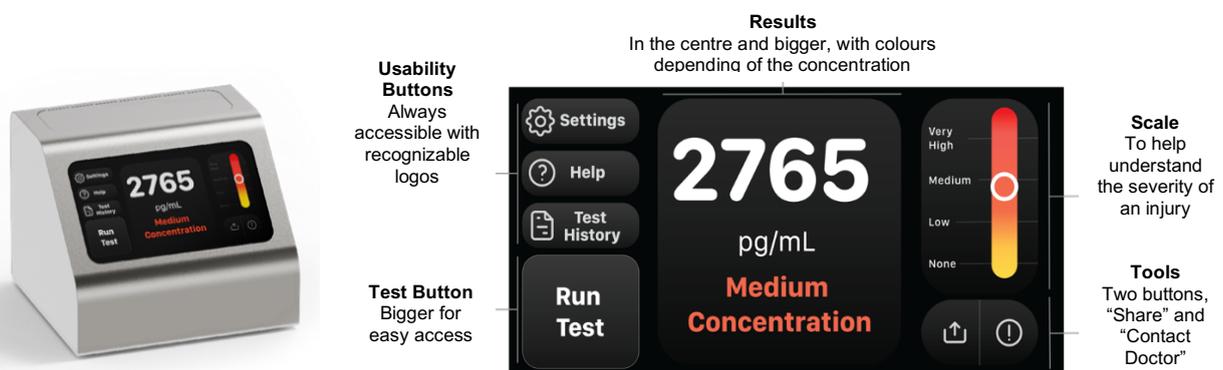
#### Software and hardware

The hardware configuration consists of a Raspberry Pi 4 (RP4) serving as the central processing unit. It communicates with various devices, including a touchscreen for user interaction, a micro-pump connected to for precise sample delivery, and an impedance analyser for EIS measurements.

The software configuration involves two threads running on the RP4. One thread controls the graphical user interface (GUI) displayed on the touchscreen, allowing users to initiate specific actions related to the system's functionalities. The other thread, the Control Thread, coordinates the micro-pump and impedance analyser operations sequentially. It manages sample delivery, initiates measurements through the impedance analyser software, and calculates GFAP- $\alpha$  concentration based on the acquired data. Further details regarding the software and hardware can be found in Appendix IV.

#### User interface

The final design of the device (depicted in Figure 5) is housed in an aluminium box (18x17x14 cm) with a user-friendly touchscreen interface. The GUI is designed for ease of use and visual appeal. The key design feature is a prominent, large-font display of the GFAP- $\alpha$  concentration at the centre of the screen, accompanied by a vertical bar indicator showing the relative amount. The indicator's position indicates the severity of the GFAP- $\alpha$  level, with color-coded visuals to highlight critical levels. Simple and clear icons are used to guide users through the device's operation.



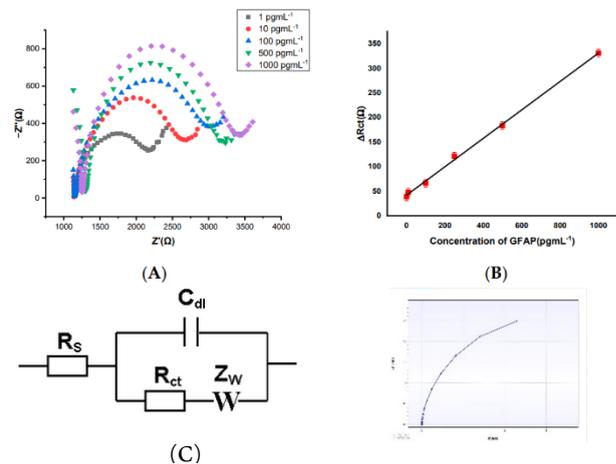
*Figure 5: 3D render of the final design of the device (left). The device is equipped with a touchscreen which the user can utilise for control. The user interacts through an intuitive GUI with a sharp design (right).*

To use the device, the user inserts the functionalized chip using a slide-in/out mechanism. The user then adds the serum or blood sample and initiates the analysis through the "Run Test" button. The micro pump pulls the sample through the chip, followed by a PBS rinse. The impedance analyser performs the EIS measurement, and the GUI updates with the measured GFAP- $\alpha$  concentration. The user receives information on the significance of the value and appropriate actions to take based on the results.

## Technological feasibility

To prove technological feasibility, three chips have been measured with each of them being submitted to different concentrations. The characteristic Nyquist plot, as seen in literature by (Ozcelikay et al., 2022), is also observed with our chips (see Figure 6). This plot is the result of the difference in ferrocene permeability through the functionalized layer. When GFAP- $\alpha$  is bound to anti-GFAP Ab, it functions as a blocking agent to the permeability of ferrocene. After this step the Nyquist plot will be analysed using a curve fit to a predetermined model, the Randles-Warburg circuit resulting in a different  $R_{ct}$ . To correct for variability between chips we need to normalise our chips by measuring a blank test and measuring the difference in  $R_{ct}$  after introduction of GFAP- $\alpha$ . This  $\Delta R_{ct}$  will vary with concentration, enabling us to make a calibration curve.

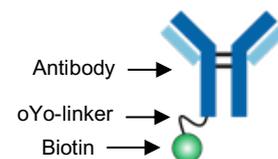
We observe a correlation between existing literature and our current results (see Figure 6D). The line that we see in the Nyquist plot is following the same trend as seen in the previous mentioned literature. To be able to build a calibration curve, an electrical model that best describes our system is fitted to the data and parameters are found. While most partners are fixed, the charge transfer resistance  $R_{ct}$ , which corresponds to the diameter of the semicircle observed in Figure 6A, is expected to increase with the increasing concentration of GFAP- $\alpha$ . This will be used for a proof-of-concept calibration curve.



*Figure 6: Working principle of the sensor found in literature. (A) Nyquist plots of different frequencies (B) calibration curve (C) Randles-Warburg Circuit (D) Proof of concept: Characterising the impedance using a redox mediator.*

## Molecular recognition

For immobilization on the surface of the sensor, the anti-GFAP Ab is biotinylated using the photo-activated oYo-linker (see Figure 7). The linker binds biotin to the heavy chain of the antibodies. This, although feasible for a small scale, would be prohibitively expensive if replicated for commercial exploitation due to the limited efficiency of oYo-linkers and high cost. Rather, our recommendation is to design a plasmid with the sequence of the antibody followed by the sequence of streptavidin protein fused to a N-terminal His-Tag, without any gap to form a recombinant antibody sequence. The plasmid can be amplified by using *E. coli* as host and translated in *Saccharomyces cerevisiae* to ensure the correct posttranslational modifications. The biotin can also be used to concentrate the protein from the cell culture.



*Figure 7: Representation of biotin conjugation to the anti-GFAP antibody through oYo-Linkers*

The sensing technology employed might not give significant difference in concentrations close to each other, which can be tackled by introducing a secondary sensing molecule. The antigen could be bound to a secondary body such as a nanoparticle of 400 nm size, which would increase the size of the entity being sensed, leading to a greater feedback signal by EIS, effectively making the technology more sensitive.

The biotinylation of the antibody was based on calculations relating to 1  $\mu\text{g}$  Ab : 1  $\mu\text{L}$  of oYo linker. Calculations for the concentration of biotinylated antibody to be functionalized on the sensor surface are based on lab optimized protocol by the Molecular Nanofabrication (MNF) lab, University of Twente. 1.5  $\mu\text{g}$  of biotinylated anti-GFAP Ab, suspended in 40  $\mu\text{L}$  of PBS is used for surface functionalization.

After establishing a protocol for the surface functionalization, the functionalization process is tested on open-cell chips, to verify that all the steps are being carried out correctly. Therefore, the reagent is pipetted on top of the chip surface and after waiting an appropriate reaction time the impedance response is measured after each step of the protocol. The amount of reagent used is in excess compared with the binding sites of the gold surface, to maximize the saturation. Specifically, 40  $\mu\text{L}$  of a 10 mg/mL solution of Biotin-400PEG-thiols, 40  $\mu\text{L}$  of 200 mM SAv solution, and 20  $\mu\text{L}$  of the antibody's solution are used on each chip. Other options considered for the surface functionalisation can be found in Appendix IV.

It is observed that there is always a change in the magnitude of the response, confirming that the EDL of the chip surface was modified (Figure 8). Note that above 100kHz, the phase plot does not correspond the characteristic shape and thus measurements above that frequency are not valid. From this, the next move was switching to close-cell chip with the proper microfluidic channels.

### Physical transduction

Firstly, the noise level of the final chip design was tested by following our measurement protocol using serum without GFAP- $\alpha$  as a sample. Furthermore, two samples of 7500 ng/mL and 2500 ng/mL were tested in the same way. This resulted an insignificant change in measurements for serum with and without GFAP- $\alpha$ . To mitigate this issue, sandwich assays with particles were used to increase the change in bulk impedance when the analyte binds to the antibody.

Notably, we address widespread challenge of non-specific bindings in real human serum and nuanced differences between analytes in standard and real human serum, bolstering our biosensor's limitations in real application.

Ideally, we would use a chip that is tailor made for our use case, as the electrode width and gap size between them can greatly influence our sensitivity. Therefore, we chose to fabricate our own chips that were designed using simulations which take our use case into account. with particle assisted sandwich assay, the grooved chips would be most optimal, since the particles will be trapped in the grooves bounded to the antibodies in 180 degrees and show higher impedance change compared to planar electrodes. Due to the use of IDE's and the desired scale of our electrodes we chose to use gold sputtering to fabricate the electrodes. This was done in combination with an etching step to create gold grooved electrodes. This proved to work adequately in our design, however, the fabrication method proved to be prone to manufacturing defects. These defects were likely caused by small dust particles that are still present in the cleanroom. These dust particles result in some of the electrodes to be connected by a tiny piece of gold, making the chip unusable for EIS. Time and resource limitations made us reconsider to shift to an off-the-shelf chip platform.

A parallel challenge arose during the bonding of the IDE surface with PDMS, where plasma treatment was employed. Prior to bonding, the impedance between the electrodes indicated an infinite separation, signifying no contact. However, post-bonding, the IDE's electrical properties shifted, resulting in altered impedance, rendering the chip unsuitable and unreliable for EIS measurements. This issue may also stem from the possibility of dust particles becoming trapped between the IDE and PDMS surfaces during the bonding process, leading to a minor electrical short between the electrodes.

### Reader Instruments

The process of serum measurement involves pipetting the serum into the container and subsequently adding PBS. Currently, the program initiates the first pump with the GFAP- $\alpha$  serum, pausing for completion before prompting the user to introduce PBS and restart the pump sequence. However, this demands precision and may pose challenges for untrained users.

To address this, an additional switch linked to an internal PBS reservoir can be integrated (depicted in Figure 9), enabling full automation. This enhancement eliminates user intervention, with the only requirement being the application of GFAP- $\alpha$  serum to the cartridge and pressing the "Run Test" button. Notably, one challenge in implementing this automation is to avoid introducing air bubbles into the microfluidic channels when switching between GFAP- $\alpha$  serum and PBS.

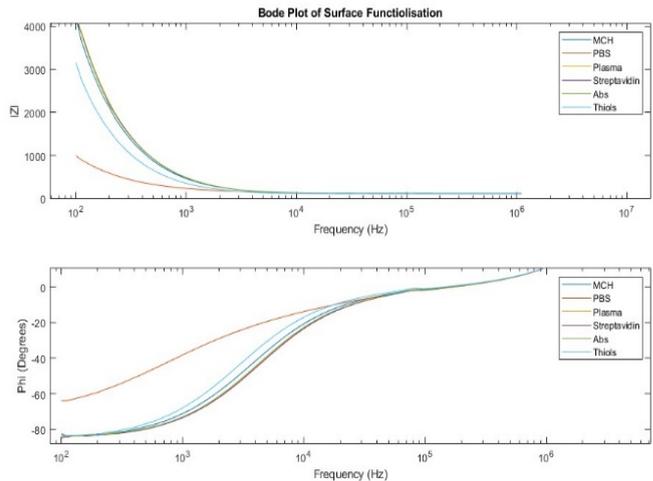


Figure 8: Bode plot of different functionalization steps

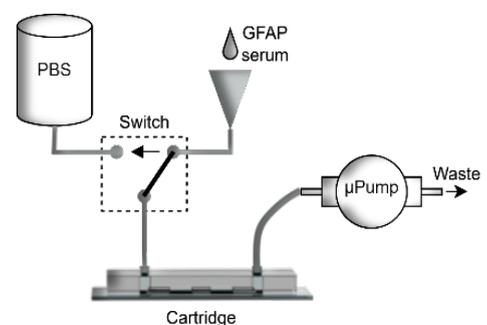


Figure 9: Full automation of the GFAP- $\alpha$  measurement

## Originality

### Team captains

Starting with literature research we have carefully reviewed several sensing methods including optical sensing – micro-ring resonators, asymmetric Mach-Zehnder Interferometer, miniature spectrometer sensing MEMS; electrical sensing – Nanopipettes with the use of aptamers, Nanocapacitor array, Ultra-micro electrodes for Cyclic voltammetry, Square Wave Voltammetry, Ion-Sensitive Field-Effect Transistor, Electrical Impedance Spectroscopy (EIS). After discussing our ideas and getting feedback from experts, we decided to go ahead and elaborate on the EIS concept. For the fabrication of the electrodes, COMSOL simulation was performed to do geometrical optimisation study for the IDE.

We are exploiting the mechanisms of surface coating chemistry for functionalising the surface of our electrodes, to finally be able to detect the GFAP- $\alpha$  protein from plasma, utilising the strong affinity of thiols to the gold surfaces in combination with biotin and streptavidin chemistry as well as oYo-Link biotinylation system. The measurements were performed and optimised using a lock-in amplifier, finding that the measurements will need to implement an extra step for more sensitive signal. For this, we decided to amplify the signal using particle labels that will bind to the analyte and this way make the chain heavier and easier to detect. For the label sandwich assay we make use of a biotinylated anti-GFAP Ab pair conjugated to a biotinylated polystyrene particle conjugated with streptavidin. For the biotinylation of the wanted antibody, we perform an NHS/EDC coupling protocol.

Regarding the reader instruments and user interface, we have pushed the boundaries by transforming the entire system into a Point-of-Care device. Our efforts encompass user-friendly design research, involving numerous iterative refinements. Moreover, we have embraced a modern user interface, deviating from conventional software paradigms. Additionally, our measurement process boasts significant automation, elevating the device beyond biochemical sophistication to technological advancement. This was done by implementing a Raspberry Pi, a touchscreen, a micropump and an embedded impedance analyser. To realise the device, custom hardware and software were developed. This way we had a chance to delve deep into industrial designing and embedded systems.

### Team supervisors

With their diverse expertise, the team began with a comprehensive literature review, delving into photonics, electrochemistry, and MEMS-based assays, later adding expert opinions to their own review. After careful consultation and evaluation of factors such as availability of expertise, device availability, and associated costs, they chose a custom-designed electrochemical impedimetric sensor. They primarily worked in the Bioelectronics Group (BE) lab at the University of Twente and fostered collaborations with the NanoLab cleanroom, Molecular Nano Fabrication Group (MNF), and Bio Systems Group (BIOS).

The team capably handled COMSOL simulations, planar electrodes design, microfluidic channel development, experiments, device assembly, and the creation of a physical product and user-interface. After fabricating a first batch of chips (Mesoscale Chemical Systems – MCS), and due to challenges related to resource and time constraints, chip fabrication was outsourced. Mohammad Saghafi provided supervision for the electrochemical impedance spectroscopy measurements, while Nico Overeem was asked for advice on surface chemistry, and Pep Canyelles Pericàs (Robotics and Mechatronics – RaM) focused on mentoring the business case and team coordination.

Despite the EIS measurements being a new terrain for them at the BE lab, the team swiftly learned its intricacies, effectively identifying and navigating potential pitfalls. Their initial findings underscored the necessity for chemical amplification, prompting a shift to a sandwich assay. For the surface chemistry, the team showed a high level of independence, asking advice when they found they needed it and implementing advice effectively without further supervision. The surface chemistry entailed a sequence of SH-PEG-biotin, streptavidin, and oYo-linkers-assisted biotinylated antibodies. This was followed by a serum flow to negate non-specific analytes, the addition of the primary analytes, and then the particles. The team utilized streptavidin-conjugated polystyrene particles, pre-coated with antibodies.

Moreover, they designed an intuitive user-device interface, leveraging a Raspberry Pi for both the EIS analyser and user interface. The project's commendable success was a testament to the efforts, in particular from the captains, and complemented by the guidance of the supervisory team.

Mohammad Saghafi

Pep Canyelles Pericàs

Diana Andreoli

Thomas Martens

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## Translation potential

### Introduction

Throughout the world, an estimated number of 69 million people suffer a TBI annually (Dewan et al., 2018) out of which up to one-third occur in sports activities, especially in high-contact sports (Theadom et al., 2020).

Within rugby the incidence of TBIs has risen significantly from four to 13 concussions per 1000 player-match hours (Oris et al., 2022). Similarly, in American football, an average of 0.61 concussions per NFL game is estimated, and over 40% of retired NFL players show evidence of traumatic brain injury (Canseco et al., 2022a). A study focusing on retired soccer players shows that 81% of the players had lasting effects like loss of concentration and memory following (unrecognizable) concussion in their career (Graham et al., 2014)

These figures are not definite estimates because of underreporting and missed diagnosis. Thus, the numbers are larger. This highlights the need for prompt diagnosis to facilitate the most effective recovery strategies in elite sports. We propose a biosensor with the ability to objectively measure GFAP-levels in the blood, which serves as an indication for TBI.

### Stakeholder desirability

#### Customers

We target high-contact sports, with a focus on rugby, American football, and soccer. The initial phase of our strategy involves targeting rugby clubs throughout Europe, starting off in Italy with the Top10 rugby league as our first phase in the research domain. Next, we will expand to the United Kingdom and France. Our business in the UK will be concentrated on the premiership rugby league, consisting of 11 teams. In France, the main focus will be the Top14. After achieving proof-of-concept in European rugby, we expand our customer base to overseas markets (US National Football League, NFL) and other sports such as soccer (US and Europe), creating a product pipeline to serve different customer segments.

#### Customers' needs

By conducting end-user interviews, we realise that customer needs of high-contact sports are akin.

The first customer need is to reduce missed diagnoses: mild TBI's are often missed during diagnosis when symptoms are atypical or late in presenting (Di Pietro et al., 2018), which was confirmed by René Hoevenaar, a sport scientist from FC Twente and lecturer at Saxion UAS (see Appendix VI). Detecting all types of TBI's on the field is beneficial to sports clubs, as TBI's result in long-term neurological deficits of athletes such as seizures, neuroendocrine dysregulation, psychiatric issues, sleeping disorders and neurodegenerative diseases (Bramlett & Dietrich, 2014). A device capable of objectively assessing TBI will aid in diagnosing all types of TBI's and reduce long-term effects.

Furthermore, the customers mentioned the need for an objective measure to differentiate the severity of TBI. Currently, Italian rugby players are side-lined for a standard four-week period after sustaining a TBI, irrespective of severity. This protocol deviates from the recommendations given in existing literature regarding return-to-play timelines (Sahler & Greenwald, 2012). The introduction of an objective measure capable of promptly classifying the severity of a TBI was described as "revolutionary" by Dr. Pasin (see Appendix VII).

Another customer need is to minimize the long-term fallout of athletes. By detecting TBI in time, the right recovery strategies can be implemented, increasing the chances of a full recovery (Li et al., 2020). This significantly reduces the likelihood of experiencing long-term impact on performance later. With early detection athletes can perform at their normal level after returning to play. Moreover, over 50% of NFL players reported not informing medical staff about their symptoms after sustaining a probable concussion at least once during their career (Canseco et al., 2022b). As athletes have a financial incentive to play, it is of importance to combine subjective symptoms with an objective measure (Ledreux et al., 2020). Symptoms of TBI can also be missed due to its masking by sedatives (Valente & Fisher, 2011). Our biosensor allows for no room for underreporting and masking symptoms after trauma to the head or collision.

Additionally, interviews with two experts associated with FC Twente and Heracles Almelo, René Hoevenaar (expert in the field of data analysis and technology implementation) and Wouter Welling (physiotherapist specialized in recovery of top athletes) (see Appendix VIII) show the needs of the soccer segment. Both indicated an interest in our sensor, and added the clubs' needs for a device with capability to measure multiple biomarkers, which our platform can supply. With the detection of other biomarkers, we can diversify.

#### Qualifications of a profitable customer

Our customer base has a high concern for player safety and performance as players are valuable assets. Currently, clubs need a blood sample test for assessing the level of GFAP and other biomarkers, which is an additional competitive advantage. Players want to know more about their health status to increase their well-being and performance, thus assuring their complicity in development phases.

## Stakeholders and their needs

We place stakeholders in the centre of our development route. By collecting their feedback early, we can validate our hypotheses, improve our value proposition, and identify new opportunities. The stakeholders' needs, as well as their role are further elaborated on in Table 1.

**Table 1 Stakeholder analysis**

Stakeholder	Needs	Role/use case
High-contact sport clubs, in particular elite or professional leagues	-Maintaining and improving athletes' health for medical and financial reasons. -Test multiple biomarkers using the same platform. -Trial period to verify efficiency and assess added value. -Device needs to be able to differentiate severity of TBI.	Direct customers of our business.
Sport physicians	-Obtain accurate and objective data to confirm their subjective assessment. -Reduce uncertainty in diagnosis.	Key opinion leaders, endorsement.
Athletes and professional players	-Device needs to be accurate and efficient to optimize return-to-play time and rehabilitation protocols.	Influencers.
Insurance companies	-Complement CT scan tests at hospitals.	Potentially paying customers.
Sport governing bodies	-Compliance with regulation.	Gatekeepers.

## Rules and regulations

Regulatory compliance is time and resource consuming in medical device development. For marketing our sensor in both European and American markets, obtaining requisite certifications is essential, namely CE, FDA. To mitigate this, we suggest starting in the research device domain, thus avoiding CE marking and reaching the market early while in development phase. The UK's regulatory landscape might align with the FDA regulations following the Brexit, this would be a fitting steppingstone as we plan to reach the US market.

## Value proposition

### Analysis of competitors

Key competitors to our business in the TBI market are analyzed in Table 2.

**Table 2 Competition analysis**

Company	Technology	Advantages	Disadvantages	Market/costs
Oculogica	Eye tracking	-Non-invasive -Portable device	-Does not detect TBI's that have no influence on eye control; not all TBI's are diagnosed. -No other biomarkers can be measured.	Revenue: <\$5M
BrainScope	Electroencephalography (EEG)	-Sensitive to subtle changes -Non-invasive -Diagnosis within 20 minutes	-No possibility to expand to other health factors, unlike blood tests - Less objective measurement of severity than GFAP measurement	Revenue: \$8M
Quanterix	Biomarkers	-Fast diagnosis -Sensitive -Company focusses on different biomarkers	- Slightly invasive - Big device compared to ours - Focused on lab environments	Revenue: \$112.9M

## Intellectual property of competitors

Presently, to the best of our knowledge, the underlying technology of our product, as well as its application are not bound under any intellectual property rights such as patents and copyrights.

## Prototype's competitive edge

Our prototype excels in user-friendliness, portability, and rapid data communication. Our competitive edge lies in detecting hard to diagnose mild TBIs and offering a versatile platform-technology to various biomarkers that will be developed in the future based on the same principles.

## Business feasibility

### Key resources

Key resources vital for business progression and potential expansions include raw materials and components for the biosensor, which are summarized in appendix IX. We plan on continuing to use the IDE chips from Metrohm. Material upscaling is feasible as we can arrange larger supply. Prototyping stage is arranged via support from MESA+, with plans to secure future sponsorships and generate revenue by venturing to the research domain before obtaining regulatory approval. We plan to functionalize the off-the-shelf chips within the umbrella of the ecosystem as a UT spinout.

## Key activities

To initiate our start-up and ensure a successful trajectory, a timeline is needed (Figure 10). We base our intellectual property strategy in cooperation with the know-how and information supplied to us by MESA+ and supporting research groups (Molecular NanoFabrication

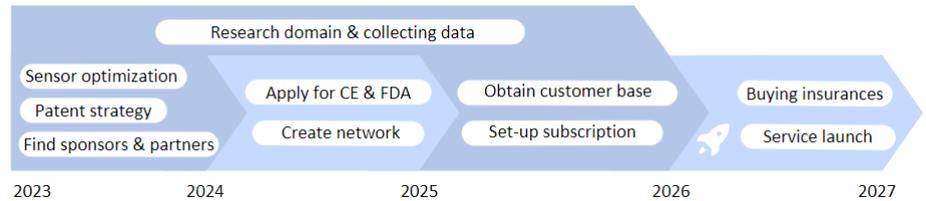


Figure 10: Chronologically ordered key activities.

– surface chemistry, BioElectronics – electrochemical sensing, Mesoscale Chemical Systems – device design and fabrication). We present a model in which we buy off-the-shelf chips from Metrohm and functionalize them with our own proprietary recipe. We also present circularity by collecting, washing and refunctionalization of the chips before re-entering them on the market.

As the chips are purchased off-the-shelf, our strategy involves patenting functionalization and reusability protocols. Our patent portfolio vision includes applications for other functionalization to detect other biomarkers. Our market strategy revolves around the sales funnel concept (Jansen & Schuster, 2011) to acquire a customer base and achieve sales. The first stage entails creating awareness about our problem-solving product through targeted digital marketing. Our digital marketing will primarily entail reaching out to our customer segment via social media channels like Instagram, LinkedIn and Facebook. In the “research” stage, the customer will learn about the added value of our sensor through various channels. As there are no companies offering services akin to ours, we expect to discover customers that will ultimately follow and purchase our services. Furthermore, to safeguard our business, we will buy goods, inventory, and transportation insurance, along with liability coverage. We start our business in the Research domain to get our product selling before getting certification. The Italian rugby market will be used for this, due to their currently existing affinity for blood tests in elite sports. After certification, we start in the UK’s and Frances commercial rugby market before further expansion to American football. Another stage follows by researching and detecting different biomarkers for elite sports assessment.

## Key partners

These partnerships are carefully cultivated to foster mutual benefits, with both parties striving for a “win-win” scenario (Table 3).

**Table 3** Key partners

Key partner	Provide	Given in return
Metrohm	- Supply of portable impedance spectroscope - Supplier of interdigitated electrode chips.	- Opportunities in new markets.
Jobst Technologies	-Supply of micropump for the device.	- Opportunities in new markets.
MESA+	-Strategic support (know-how, personnel)	- Brand awareness.
University of Twente	-UT spin out support.	- Kudos. Financial return.
TechMed Centre	-Guidance in medical device regulation.	- Brand awareness.
Novel-T (UT TTO)	- Guidance in route to market and investments.	-Brand awareness.
Demcon	- Instrument manufacturing.	- Partnership or supplier.

## Sustainability

Circularity is central within our company. The compact nature of the instrument translates to less resources needed for manufacturing (dimensions: 14 X 18.5 X 17 cm). We will refurbish the chips by separating the functionalization layer from the electrodes. In this way we can reuse the chips. The final product is planned to get rid of any moving parts by replacing the microfluidic pumps generated pressure-driven fluid flow by a capillary-force-driven one, removing any moving parts. Modularity and reparability are taken into consideration: The instrument is easy to open to swap out the defective bit.

## Financial viability

### Costs projection

Cost projection is separated into two parts: the portable biosensing instrument (a) and the biosensing chips/cartridges (b).

(a) Biosensing instrument. We will scale up our prototype with partnerships with established manufacturers, for instance with Twente based (and UT spin out) Demcon. An extended breakdown of the costs per chip and the device itself can be found in appendix IX.

(b) The cartridges need interdigitated electrode chips (IE’s) as basis for functionalization. Our current partner Metrohm supplies these electrodes to us with a volume discount of 20% for batches of 40. Using bulk buying and negotiations, the best-case cost for the IE’s will come down to €22.40/IE. The chips can be refurbished and cleaned using a piranha base solution, which costs €0.10 per chip for cleaning. Transport costs will also need to be considered. However, these are not determinable at this point due to them depending on multiple

uncertain factors, such as, location of company, number of customers, and location of costumers. We expect to make 10 devices per batch, but due to volume discounts materials are to be bought for long term manufacturing in batches of 100 units.

These however will not amount to all our expenditures. We will also have costs regarding our research, development and implementation outlined in the following table 5. An explanation of the cost categories can be found in appendix X.

### Sales price

Due to the insights of Dr. Pasin it is known that on average he receives 10 visits from rugby players in a week, with each appointment costing 200 euros. This is from 1 rugby club. These visits, however, do not comprise solely of head injuries. Using the information that in rugby over 25% of injuries are to the head/neck region (Gabbett, 2000). And thus, would have benefits testing for TBI we can calculate that the minimum weekly costs of TBI for an Italian rugby club is 500 euros. This means clubs are spending €2000 a

month currently on TBI related injuries. We use this to benchmark the research domain market, estimating that we can charge €1000 per month during the development phase. With this, 10 cartridges are provided as a standard or more depending on demand. The price of the service plan will rise according to demand.

Due to the insights of Dr. Pasin it is known that on average he receives 10 visits from rugby players in a week, with each appointment costing 200 euros. This is from 1 rugby club. These visits, however, do not comprise solely of head injuries. Using the information that in rugby over 25% of injuries are to the head/neck region (Gabbett, 2000)

### Market analysis

The early adapting markets are Italy, France and the UK. In table 7 their respective data on the market is noted. The UK is the second largest Rugby playing country with France being the number one. Italy comes in at number 10. The NFL itself is a lot smaller in numbers of clubs and players but generates significantly more income. To give further incentive to develop our business in the latter market.

**Table 6** Market size of rugby and NFL. Ref: (OnRugby, 2023; Rugby Football Union, 2023; ZoomInfo, 2023)

Country/Market	Registered Players	Profit Federation	Average Revenue top Clubs
UK Rugby	276,000	17 million	€21 million
Italy	80,000	1.5 million	No Data
France	360,000	10 million	€34 million
American NFL	1,700	9.9 billion	€800 million

### Market outlook

In interviews with our stakeholders, we concluded that the ability to measure different biomarkers that give useful information in sports context would be a huge benefit to the product. Think of lactate, glucose, testosterone, hemoglobin and more. This expands our market size drastically, since our product will no longer only be used in high physical contact sports, but also in athlete performance analysis, including AI analytics. This is used in almost every top-class sport. By collecting the data of the tests, we can keep track on the amount of TBI's or other biomarkers within a certain field of sports or specific sports teams.

### Golden standard

Currently the golden standard for measuring TBI is diagnosis by medical staff or hospital staff on base of the Glasow Coma Scale and/or MRI/CT scans. All treatments are the same, regarding of severity. These current methods make it hard to detect mTBI, which is the biggest portion of all TBI's comprising of at least 80% of all cases (Levin & Diaz-Arrastia, 2015). Due to the often-absent visible brain damage and lack of loss of consciousness associated with most cases of mTBI in sports.

### Selection of benchmark

Our main market is detecting mTBI. Due to its nature, they currently have no current benchmark in the market. In general, TBI's are solely diagnosed in a clinical setting with the help of a medical professionals and CT/MRI scans. The use of CT/MRI by medical professionals to determine if a sport related TBI has occurred will be our benchmark.

**Table 4** Device and chip cost/unit

Cost/unit	Cost, EUR
Device cost	310
Chip cost	~35

**Table 5** Research, development and implementation costs

Cost Category	Cost, EUR
R&D Expenses	1.000.000
Regulatory Compliance	3.000.000
Intellectual Property (IP) Protection	500.000
Manufacturing Setup	100.000
Marketing and Distribution	100.000
Total Cost, EUR	4.700.000

## Justifying costs and generating sufficient revenue

Out of 82 clubs competing in top Italian rugby we estimate that around 20 clubs will be willing to start a partnership to get more insight in their players health. The monthly revenue is calculated in table 7. These numbers give us a yearly revenue estimate of €156,000 a year (this is with costs of the chips already subtracted). This will not be sufficient to cover all development costs but helps paying back investors during development with a yearly return of 3.3% (based on the development costs and investment of €4.7 million).

**Table 7** Costs and revenue analysis per club per month

Revenue Category	Per club, EUR	Amount	Total, EUR
Chips*	-350	20	-7,000
(Device)	-310	20	-6,200
(Device deposit)	310	20	6,200
Service fee**	1.000	20	20,000
<b>Total</b>	<b>650</b>	<b>20</b>	<b>13,000</b>

\*based on 10 visits a month  
 \*\* based on 50% clubs already spent on head injury diagnosis

## Revenue streams and business strategy

We opt to go for a service fee-based revenue stream. We lend the device to our customers for use, and they pay a monthly fee for our service. This includes the renting of the device, servicing to keep it functional, the supply of chips and their collection once used. To appeal to more customers, we aim to divide our service into different plans. All will have a fully functional device, but with a better subscription plan you get extras. Such as priority with servicing and supply of new chips. With a greater subscription plan, we can feedback data analysis to the clubs to give them a competitive edge by having more knowledge of the other teams. As our product develops, we aim to have our platform-technology suited for multiple biomarkers.

### Comparison between expected revenues and costs of development.

As calculated before, we will make revenue of 650 euro per club in the development phase. For estimation, we keep prices the same as we reach other countries, we can generate €117,000 in the UK and €85,800 in France and €156,000 in Italy after 3 years of development.<sup>1</sup> After getting into the commercial market, it is likely that more clubs get interested instead of the expected 1 in 4 of the top leagues. Increasing our pricing for the different service plans also increases our revenue.

### Break-even point

The first three years our revenue will solely come from sales in the Italian research domain. After three years the UK and France markets will be reached. After a total of five years, we expect the value of the device being recognized through the whole rugby market which will give an increase in market to all rugby playing countries and increasing clubs within the current countries as well with an estimated 15% growth per year. Which ends up getting us breaking even after 10 years with an average revenue of €1 million (Appendix XI). Note that this is the minimal break-even time, since this calculation is based on the basis service fee of €1000,- a month. When rolling out in the commercial market our product price will increase with different plans.

## Business intelligence

We will have all devices equipped with the capability to store data. This data can be collected by our company and gives us a good insight in the amount of TBI's happening. In the development phase, we can also compare these results with official medical results in cooperation with the clubs and hospitals. This gives good information on the incidence of TBI and gives us data that can help us get our product certified. Using data from every club we can use this as another product we can conclude in our service plans as stated before. Additionally, we will be aligning with the European General Data Protection Regulation (GDPR) to ensure safe handling of personal data.

<sup>1</sup> Based on the assumption 1 in 4 top level clubs will adapt the device. Amounting to France: 11 clubs; UK 15 clubs

## Team and support

### Contributions of the team members

**Diana Andreoli** – Team Captain, Biomedical and Electrical Engineer. Development of the device and its sensing platform, composing protocols and working on the lab. Appreciated team player and leader. Key connection with supervisors and external communication together with Thomas Martens.

**Elena Antonioli** – Chemical / Materials science Engineer. Developed the surface functionalization protocol and took part throughout the lab work for preparing reagents and PDMS parts, assembling, and testing.

**Pablo Jiménez Chillón** – Industrial Design Engineer. Designed the prototype of the device, the user interface, and defined the way users interact with the device. Responsible for building and assembling the final device, integrating all the parts into a portable, easy-to-use, and easy-to-repair format.

**Roman Koval** – Advanced Technology Engineer. Worked on biosensor platform conceptual research and proof-of-concept laboratory work and signal transduction. Developed COMSOL simulation for non-planar IDEs geometrical optimization. Designed the non-planar IDE chip (mask design and facilitated the interaction with clean-room staff). Is the main team member performing the EIS measurements.

**Thomas Martens** – Team Captain; Biomedical Engineer. Focus on the business case development, translating a technical product to a business case; also worked as lab support. Managing customer discovery and feedback collection as well. Key connection in communication together with Diana.

**Mīkelis Putnieks** – Product Design Engineer and business case developer. Designed the device and cartridge technology, including PDMS microfluidics. Focused on evaluating the financials of business case.

**Thomas van Poppel** – Chemical science Engineer. Delved deep into the concept development of the sensor, composing lab work protocols and carrying out some experiments.

**Jan Pieter de Rie** – Embedded System Engineer. Specializing in converting complex systems into a user-friendly Point-of-Care device by skilfully utilizing Raspberry Pi control, optimizing functionality, and integrating various components seamlessly.

**Rory Timmerman** – Advanced Technology Engineer. Focused on the biosensor platform, processing the data, and proof-of-concept laboratory work. Worked out and optimised protocols for EIS measurements, prepared essential device components and is the main team member performing the EIS measurements.

**Vishal Tuli** – Biotechnologist/Bioengineer. Focused on surface functionalization of the sensor and molecular interactions between the functionalized molecules with proof of concept for the interaction strategy. Took part in assembling the Chips with PDMS and testing them.

**Maud Westerbeek** – Business case developer and head of social media. Specialized in translating the biosensor to a suitable market. Managing our social media platforms and providing regular updates about our achievements.

### People who have given support.

**Dr. ir. Pep Canyelles Pericàs** – guiding and coaching the team, giving insightful feedback on both the technical side and the business case, where he helped the team to find the niche market application. Pep held regular weekly meetings and was in charge of overall team coordination and house-keeping.

**Mr. Mohammad Saghafi MSc** – Daily (lab) supervisor, aided in understanding the electrochemical sensing concepts and guided the team strategy. Held daily stand-up meeting to and provided critical feedback.

**Dr. ir. Nico Overeem** – Helped us understanding the surface chemistry, provided us with initial lab material and IDE electrodes. Reviewed our work and gave us constructive feedback.

**Dr. Sevil Sahin** – Helped us determining the best anti-GFAP immobilization protocol, optimising the cleaning protocol as well as reviewing our report and giving us final feedback.

**Dr. Jannis Schlicke MSc** – Providing help executing the experiments, guiding us in the chemistry lab.

**Mr. Stefan Schlautmann** – Guided the non-planar IDE design and developed fabrication protocol. Fabricated proof-of-concept IDE chips in the MESA+ cleanroom.

**Ms. Daria Bugakova MSc** – Aided in the milling of PMMA molds used for our microfluidic parts of the chip.

**Dr. Mariia Zakharova-Kolezhuk MSc** – Helped us understand the lock-in amplifier instrument used for EIS.

**Dr. Suryasnata Tripathy** – supported us in lab work, understanding the concepts and acquiring results.

**Prof. Dr. Serge Lemay** – provided us with lab space at the BioElectronics group (BE), where we could use the lab equipment and lab material. **Ing. Ab Nieuwenhuis** – placed orders and followed up on them.

### Sponsors and partners

**Mesa +**, contact person: **Prof. dr. ir. Loes Segerink** - Our partner from the University of Twente, which supplies us overall support (budget, access to know-how of collaborating groups) with Prof. Segerink as our senior supervisor and the contact person.

**Metrohm**, contact person: **Mr. Martijn van der Plas MSc** – Supplied us with gold plated electrodes with a discount and provided us with a small portable impedance spectroscopy ( $\mu$ Stat-i 400).

**NovelT**, contact person: **Dr. Esther Rodijk** – provided feedback on the business case; **Rogier de Haan** - provided feedback on the business case.

Contact person:

**PalmSens**, contact person: **Mr. Lutz Stratmann MSc** – Provided with support on small impedance analyser.



## Final Remarks

We would like to express our sincere gratitude to all those who have generously contributed their time, expertise, and support over the past year. Your invaluable assistance has played a crucial role in our progress and achievements. Your guidance and encouragement have truly made a difference, and we are deeply thankful for your unwavering commitment. Thank you for being an integral part of our success.

It has been a remarkable journey collaborating with this exceptional team on our project. Our collective growth has surpassed even our initial expectations, encompassing both the refinement of technical proficiencies and the nurturing of essential interpersonal skills. Having a team composed of students from multidisciplinary backgrounds has broadened our perspective and brought diverse knowledge from different fields to the table.

The team firmly believes that the concept we've explored harbours substantial untapped potential, ripe for further exploration through subsequent research endeavours. In the near future, we extend the development of the biosensor beyond the competition, improving on the prototype and seeing the potential of bringing our device to the market as well as expanding its application for more than TBI detection. With more time and resources, we will be able to identify the limit of detection and the saturation capacity of the sensor and its validity. Alongside we are eager to set-up a student team organisation dedicated to the SensUs Competition.

Enhancing the efficacy and reproducibility of the sensor remains an enticing prospect. Expanding the scope of experimentation with surface chemistry, fine-tuning EIS measurements, and optimizing the sensitivity are avenues that hold promise. The journey towards more automated device and streamlined cartridge design also beckons as a fertile ground for future innovation.

This collective effort stands as a testament to the power of collaboration and shared aspirations, underscoring the significance of united dedication in propelling our project to its current accomplishments. As we navigate the next phase, we are fuelled by a profound sense of purpose and a collective vision, and we eagerly anticipate the future possibilities that await.

We would like to express our deepest appreciation to our dedicated supervisors and mentors, Dr. ir. Pep Canyelles Pericàs and Mr. Mohammad Saghafi MSc, who have given us endless support, guidance and valuable feedback throughout this past year.

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## Appendix I: Targeted epitopes of GFAP

Antibodies used are human IgG1 monoclonal antibodies produced in mice. This indicates that the antibodies used in the study are monoclonal antibodies (mAbs) of the human IgG1 isotype. These mAbs were produced in mice through a process known as hybridoma technology, where mouse B cells are fused with myeloma cells to generate immortalized cell lines that produce specific antibodies.

Supplier	Antibody Variants
HYTEST	GFAP15cc, GFAP81cc, GFAP83cc, GFAP94cc, GFAP98cc

This table specifies the source of the monoclonal antibodies and provides identifiers for different antibody variants. "HyTest" refers to the manufacturer or supplier of the antibodies. The identifiers (GFAP15cc, GFAP81cc, etc.) represent different clones or variations of antibodies that target different regions or epitopes of the Glial Fibrillary Acidic Protein (GFAP) molecule.

The listed antibodies were tested for their GFAP capturing efficiency as can be seen in the figure below and antibody 83 performed the best with minimal standard deviation and thus was used for the functionalization onto the sensor.

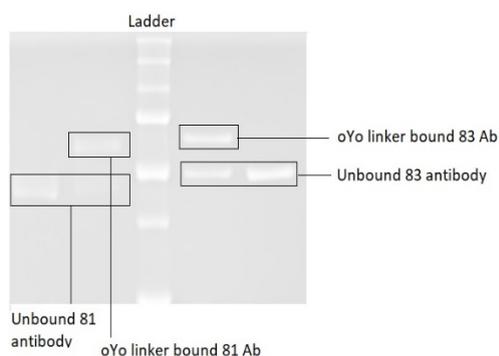
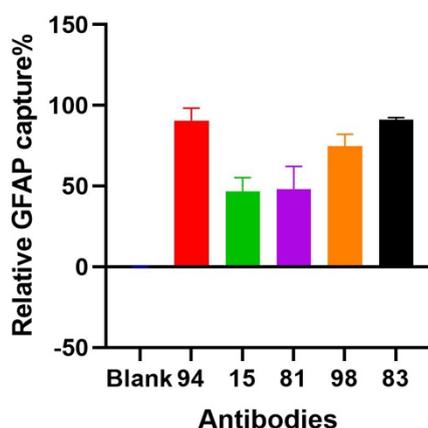
The protocol followed for this is listed as follows:-

1. Make a solution of 20ug/ml for the GFAP antigen and pour 200ul of it in the wells of Maxisorp plate
2. Incubate overnight at 4 degree Celsius
3. Next day, take the plate out and wash wells with 200ul 1x tween in PBS, thrice
4. Add 200ul of BSA Blocking buffer to each well (1% BSA in PBS)
5. Incubate for 1 hours at room temperature
6. Add 100ul of 200ng/ml primary antibody (antibody to be tested) to the wells and incubate for 1.5 hours
7. Wash the wells with 200ul 1x tween in PBS, thrice
8. Add 100ul of 300ng/ml secondary antibody, conjugated with HRP to each well and incubate for 1.5 hours
9. Wash wells with 200ul 1x tween in PBS, thrice
10. Add 100ul of TMB substrate to each well and wait till the blue colour saturates in one of the wells
11. Add 50ul of 10% 1.8M sulfuric acid to stop the reaction
12. Quantify the reaction using a microplate reader at 450nm

Primary antibody – Mouse anti human GFAP Ab

Secondary antibody – Goat anti mouse HRP conjugated Ab

### Efficiency of antibodies to capture GFAP



The biotinylation of the antibodies was tested for by SDS PAGE and it clearly shows in the figure below that the reaction was a success



## Appendix II: EIS

EIS frequency spectra exhibit three distinct regimes (Saghafi et al, 2023). In the lower frequencies the capacitive nature of EDL poses the largest contribution to the electric response. The capacitive regime is extremely sensitive to conditions as minor as molecule orientation or surface contaminations within the Debye volume. Furthermore, our surface chemistry extends significantly beyond the Debye length, placing changes at the bioreceptor layer too far from the surface to influence the EDL.

For frequencies higher than the first cutoff, the EDL capacitance contribution diminishes while the contribution of solution resistance becomes significant, which can intuitively be understood as a result of the decreasing time available for EDL formation. The second regime is also referred to as resistive and is thus easily located in the bode plot as a region with frequency invariant impedance. For frequencies exceeding the second cutoff the impedance continues to decrease. The third regime is not observable due to the frequency limitation of measuring setup available.

The inner diffusion layer of the EDL is approximately equal to the Debye length (Kemp, 2021). Our surface chemistry extends beyond the Debye length where the electric field is typically weak due to the ionic screening (Kesler et al, 2020). The inner diffusion layer is expected to be thicker than that expected from the Debye length due to the high density surface functionalization. The Debye length can further be extended through the miniaturization (Kesler et al, 2020) of the Debye volume through employment of non-planar electrodes of concave geometries.

In summary, the capacitive regime displays increased sensitivity to surface interactions but lacks adequate responsiveness to the target present on the surface functionalization. Therefore, the resistive regime emerges as a more suitable choice. Nevertheless, it's essential to circumvent areas equivalent to the electrode dimensions, approximately 5  $\mu\text{m}$ . As a result, our approach is geared towards the low-frequency edges of the resistive range, where the effects from the capacitive regime persist but are considerably subdued.

### Appendix III: Manufacturing IDE

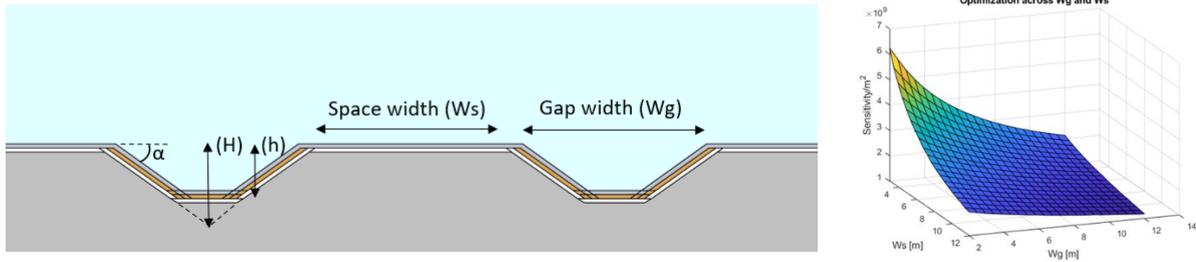


Figure 11: Geometrical optimization study of grooved, non-planar IDEs through COMSOL Multiphysics simulation.

The effects of varying Interdigitated space width ( $W_s$ ) and electrode gap width ( $W_g$ ) in the ranges of 1-6  $\mu\text{m}$  and 2-12 $\mu\text{m}$  respectively through a COMSOL simulation are shown in figure 11. The sensitivity is evaluated through the change in resistance due to the change of conductivity at the surface. The simulation showed a non-linear increase of sensitivity for a decreasing  $W_g$  and  $W_s$ .

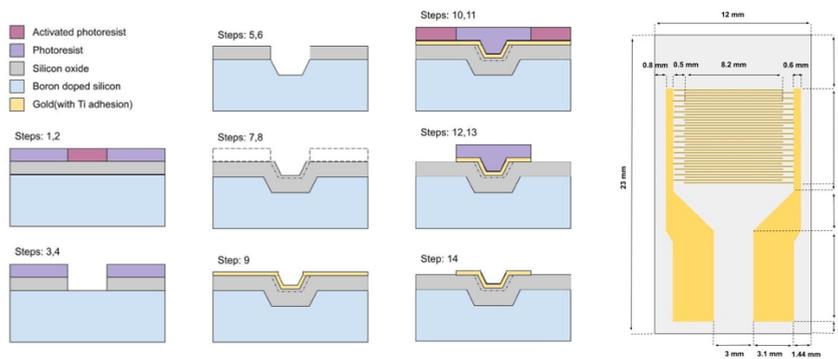


Figure 12: Fabrication steps of grooved IDEs.

The optimal IDE geometry of 1000 grooved fingers (digits) of  $6\mu\text{m}$  wide and with  $4\mu\text{m}$  inter-digit spacings were fabricated. The fabrication steps are summarised in figure 12. SEM images of the chips are shown in figure 13.

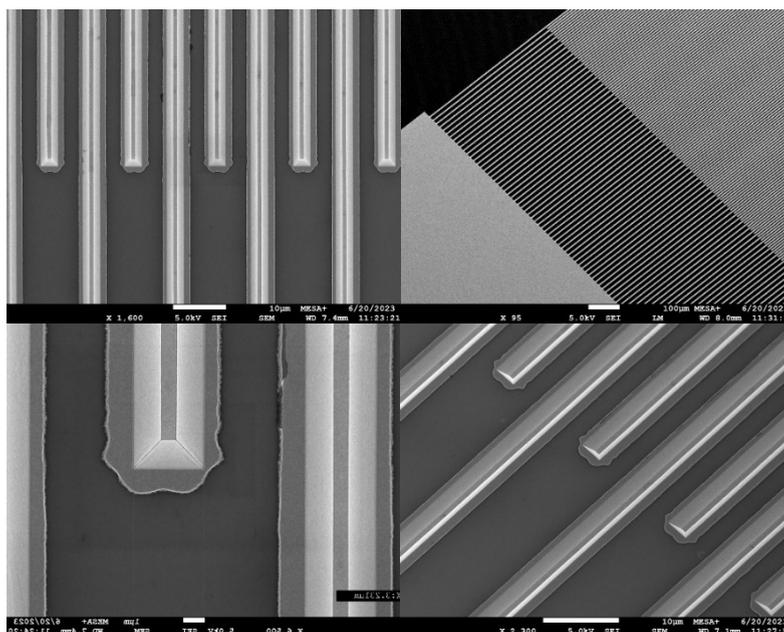


Figure 13: ESM images of the fabricated IDEs.

## Appendix IV: Surface functionalization protocol – discarded options

To establish the surface functionalization protocol, a few options were explored theoretically.

The first idea was to use aptamers: they are nucleic acids fragments able to bind to a specific protein. This option excludes the usage of antibodies. It was verified that specific aptamers for GFAP exists, but the kind of bond provided by them in the medium of blood serum could not have been specific enough. Therefore, antibodies were preferred to achieve the necessary specificity in the binding of GFAP.

To connect the antibodies to the chip surface it was, in the beginning, thought of exploiting the negative charge of the antibodies. The chip surface can first be coated with a few layers of polyelectrolytes multilayers (PEM) that would end with a positive charged layer.

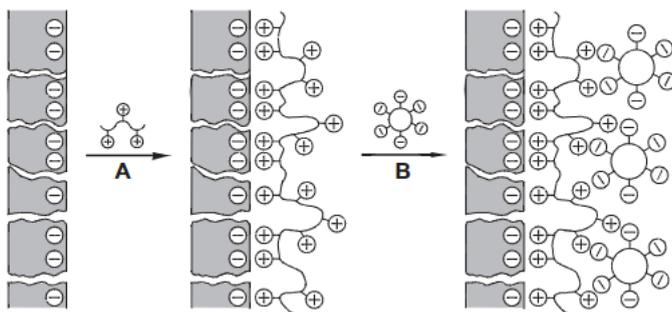


Figure 11: <https://onlinelibrary.wiley.com/doi/10.1002/adfm.200400223>

As portrayed schematically in the figure above, the antibody that is negatively charge would interact electrostatically with the coated chip surface and as such it will be immobilized on the surface.

In this way, we would avoid the problem of adding biotin to the antibodies, but the option was discarded for other possible issues. Namely, the electrostatic interaction might not be resistant enough withstand strong elution, making the chip single use. Our chips for now are still single use, but for a future prospective it is not preferable. Furthermore, the serum medium has a lot of other macromolecules that could interact with the charged layers, increasing the noise.

In the end, it was chosen the functionalization protocol mentioned in the previous section, where the click chemistry of thiols and gold along with the SA<sub>v</sub>-Biotin chemistry, were exploited to achieve a successful coating.

## Appendix V: Hardware/Software Configuration

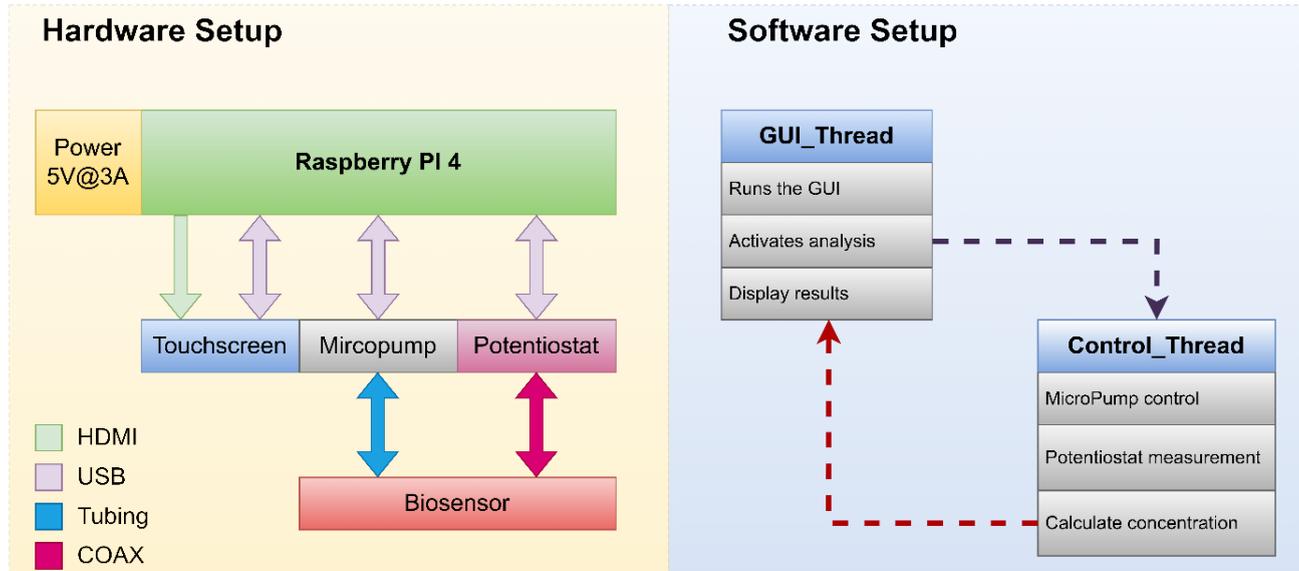


Figure 12: A schematic overview of the hardware setup (left) and the software setup (right) for the final Point-of-Care device. The left diagram depicts the interconnection of various components and their respective interactions within the system. The right diagram shows the approach to segregate the graphical user interface (GUI) and measurement control into different threads, ensuring efficient and independent handling functionalities for enhanced system performance.

Component	Model/Type
<b>Main Processing Unit</b>	Raspberry Pi 4B
<b>TouchScreen</b>	Waveshare 7 inch HDMI QLED Display 1024*600 pixels with Touchscreen
<b>Micro-pump</b>	Jobst Technologies CPP1-180-ZM Peristaltic micro pump
<b>Impedance analyser</b>	PalmSens EmStat Pico Development kit

### Main Processing Unit: Raspberry Pi 4B

The central processing unit of the system is a Raspberry Pi 4B, which serves as the core controller. This versatile and capable unit facilitates seamless communication and coordination of various functions within the biosensor setup.

### Touchscreen: Waveshare 7-inch HDMI QLED Display

The user interface is provided by a high-resolution 7-inch HDMI QLED touchscreen display from Waveshare. With a pixel density of 1024\*600, this touchscreen enables intuitive and user-friendly interaction with the biosensor's functions.

### Micro-pump: Jobst Technologies CPP1-180-ZM Peristaltic Micro Pump

The microfluidic system is powered by the Jobst Technologies CPP1-180-ZM peristaltic micro pump. This precision pump ensures controlled and accurate fluid flow through the microchannels, a critical aspect of the biosensor's functionality.

### Impedance analyser: PalmSens EmStat Pico Development Kit

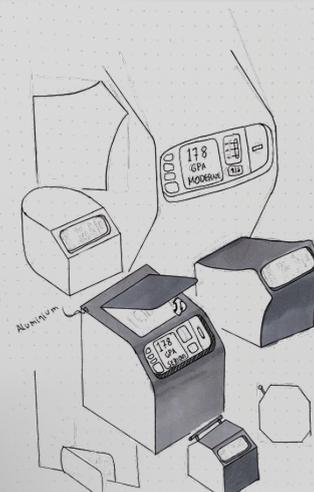
The impedance analyser component is realized through the PalmSens EmStat Pico Development Kit. This advanced kit provides the necessary tools for electrochemical measurements, contributing to the accurate and reliable analysis of sensor responses.

## Appendix VI: Design Iterations

Final design:



Different shape iterations:



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## Appendix VII: Interview profile sport scientist

### Interviewee profile:

Name: René Hoevenaar

Function: First team sport scientist

Organization: FC Twente

### Key questions and answers:

1. What is your expertise?/ what is your role within FC Twente

Sports scientist measures daily

Wellness questionnaire before training:

- Muscle soreness
- Sleeping how long / Quality?

During the training measure:

- How many km do they run / how fast(-> influences muscle damage)?
- Heart rate

Jump test + sprint test, shuttle run test

2. How do you see the data driven approach in sports nowadays, is it used a lot or still new?

Wellness reports are used to see how players recover

Some clubs use blood testing to manage recovery

3. Do you feel that TBI is a common injury amongst soccer players?

Only the bigger ones – head to head

End of the season = three of which two were in the same match

Only the bad ones are diagnosed

4. It seems you have a lot of contact with the players, do you think they are willing to test after every big match? Or even after an impact

- a. 1 our after impact / match. Test takes max 5 min till result. We take some blood.

If the goal is really clear, and if it is better for the future and your whole life. Probably only after something happened so not every day or every game

Players are always afraid of losing the spot in the team when they do not play

5. Do you think a device like ours is one that FC Twente would be interested in?

Depends on a lot of factors:

6. Do you have an estimation of how much money FC Twente for example would be willing to spent?

Depends on what the benefits are. The club itself does not have that much money.

It will be far more attractive if we are able to measure multiple biomarkers. If the sensor is a "schakel" between

A player can go to the MRI within 48 hours.

Check assumptions, for example if players will rest with mild symptoms

Notes on the meeting:

Muscle damage extension on ensor

Look into whether we can make it an active indicator – measure two days later and see if they can play again.

See if we can indicate recovery time



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## Appendix VIII: Interview profile medical professional

### Interviewee profile:

Name: Dr. Federico Pasin

Function: Medical doctor & Team doctor

Organization: Hospital of Parma & Rugby Colorno Club

### Key questions and answers

1. What is your expertise?/ what is your role within Rugby clubs

Dr Federico Pasin

He is a doctor specialized in internal medicine and urgency. Has more than 10 years experience in rugby clubs as a "field doctor". He handles athletes [low level professionals] both during the week and matches.

2. How do you see the data driven approach in sports nowadays, is it used a lot or still new?

The scientific approach is fundamental to maximize results, from prevention, handling, preparation and monitoring. In some fields there is more data driven approach than others (nutrition is high for example) but in sports it is not adequate yet especially regarding the safety of players.

3. Do you have experience using blood tests?

Yes he works everyday with it. In university he did research on vitamin D value in rugby players.. there are some papers. Rugby players are tested regularly to detect alterations of values but related to hemoglobin or electrolytes never for injuries.

4. Do you feel that TBI is a common injury amongst rugby players [or other sports you are involved in]?

TBI is very common in contact sports, it is one of the main injuries.

5. Do you think they are willing to test after every big match? Or even after an impact. Even if they don't really feel anything?

Yes absolutely. It is important to test even if there was not an impact because TBI can also happen for quick deceleration or knockback(\*) (rebound impact). Players would of course be willing, they are already monitored a lot. With covid they were tested before every match.

a. What is the normal protocol when a player is suspected with TBI?

There is a protocol provide by the world rugby federation. It is identified with neurological exams, radiological, or magnetic resonance. Blood tests are not used! There is always also a validation from a doctor that does speech, memorial tests.

b. Are players aware of the risks of TBI? And do you think they also want to have their blood tested

Not fully. When a player has TBI is stopped for 4 weeks. Continuously tested after 6h, 1 day etc... and at the end another assessment with a doctor separated from the sports club.

6. Who decides to play in the field, how is it with pressure for example a big game. Will people take their rest or not?

The match doctor of the club is the one that decides. This means that it can be a subjective opinion and can be influenced by the pressure of the game (a really good player that has no good subs). Athletes not always take the rest willingly.

7. Do you think a device like ours is one that big clubs might be interested in? Is it feasible to get on the market?

Yes of course but only with scientific validation, it needs to be reliable. Athletes could also be willing to provide their blood for research (see paper), especially if it helps for prevention of serious consequences.

It is really difficult to distinguish a normal impact from TBI, is a gray area in between, a test that can reliably tell you if there is or isn't TBI would be revolutionary. Another important thing is the distinction between low-medium-severe TBI, right now the protocol is the same for every level but if the level was known it could be treated accordingly. So far TBI is only a clinical diagnosis.

Another important factor is the individuality of the results: it could be possible to compare the sample of the same person before and after the accident to get more accurate results.

a. Do you think sport clubs/ physiotherapists would be more interested in a device as ours if it can measure multiple biomarkers? E.g. glucose levels

yes of course but it would need to be proven and reliable.

b. Do you have an estimation of how much money a club would be willing to spent?

Depends on results, if reliable they would be willing to pay. Already many clubs do weekly test that are 200€ each, so a the would be willing to spend a good amount for a device like this. It would be good also if before buying it there could be a trial period to verify the efficiency of the device and then invest in it.



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## Appendix IX: Interview profile physiotherapist

### Interviewee profile:

Name: PhD Wouter Welling

Function: Sport scientist & lecturer

Organization: Pro-F Physiotherapy (associated with FC Twente & Heracles Almelo) & Saxion University of Applied Sciences

### Key questions and answers:

1. What is your expertise?/ what is your role within FC Twente  
Sports scientist measures daily  
Wellness questionnaire before training:
    - Muscle soreness
    - Sleeping how long / Quality?During the training measure:
    - How many km do they run / how fast(-> influences muscle damage)?
    - Heart rateJump test + sprint test, shuttle run test
  2. How do you see the data driven approach in sports nowadays, is it used a lot or still new?  
Wellness reports are used to see how players recover  
Some clubs use blood testing to manage recovery
  3. Do you feel that TBI is a common injury amongst soccer players?  
Only the bigger ones – head to head  
End of the season = three of which two were in the same match  
Only the bad ones are diagnosed
  4. It seems you have a lot of contact with the players, do you think they are willing to test after every big match? Or even after an impact
    - a. 1 our after impact / match. Test takes max 5 min till result. We take some blood.If the goal is really clear, and if it is better for the future and your whole life. Probably only after something happened so not every day or every game  
Players are always afraid of losing the spot in the team when they do not play
  5. Do you think a device like ours is one that FC Twente would be interested in?  
Depends on a lot of factors:
  6. Do you have an estimation of how much money FC Twente for example would be willing to spent?  
Depends on what the benefits are. The club itself does not have that much money.  
It will be far more attractive if we are able to measure multiple biomarkers. If the sensor is a "shake!" between  
A player can go to the MRI within 48 hours.  
Check assumptions, for example if players will rest with mild symptoms
- Notes on the meeting:  
Muscle damage extension on ensor  
Look into whether we can make it an active indicator – measure two days later and see if they can play again.  
See if we can indicate recovery time

## Appendix X: Cost analysis

A breakdown of the various per-use costs of the device (labor assumed 20 EUR/h):

Cost Category	Cost, EUR	Amount	Unit Cost, EUR
<b>Chip Parts and Assembly Costs</b>			
Interdigitated Electrodes	448.00	20	22.40
Sylgard PDMS	222.00	1kg (≈500)	0.44
Labor – PDMS Casting	40 (2h)	20	2.00
Labor – PDMS Bonding, Chip Assembly	20 (1h)	20	1.00
Syringe Sample Inlet Tips			
3D Printed Chip Hold	0.06	1	0.06
<b>Chip Functionalization Costs</b>			
Streptavidin	500	(40uL per unit)	
GFAP-Antibodies	700	(2uL per unit)	0.42
oYo-Linkers	600	(1.5ug per unit)	4.95
Thiols-PEG400-biotin	480	40uL of 0.8mg/ml	
MCH	75	5mL (40uL)	
Solvents: MilliQ, PBS	100	(2mL per unit)	
Cleaning: Acetone, IPA, Toluene, MilliQ	65	1L (50mL per unit)	
Labor – Functionalizing, Quality Control			
<b>Operational Costs</b>			
Packaging	5	20	0.25
Delivery (Cold)	20	20	1
Transportation Insurance (?)	5	20	0.25
<b>Total Unit Cost, EUR</b>			

The following is a breakdown of the various costs for manufacturing a TwentUs biosensing device (labor assumed 20 EUR/h):

Cost Category	Cost, EUR	Amount	Unit Cost, EUR
<b>Device Manufacturing Costs</b>			
Custom EIS Circuitry	≈ 100	1	100
QLED Screen	2000	100	25
Shell (Laser-Cut Aluminum, 3D-Printed Plastics)	≈ 10	1	10
Microfluidics Pump, Pump Driver, Tubing	In final product replaced by capillary driven flow.		
Internal Structure (3D-Printed Plastics)	1	1	1
Raspberry Pi 4 Inbuilt Computer	5300	100	53
Chip Connecting Clip (Plastic, Pogo Electrodes)	0.5	1	0.5
Waste Container	0.5	1	0.5
USB female socket, internal wiring, and cables	1000	100	10
Labor – Assembly	80 (4h)	1	80
<b>Operational Costs</b>			
Packaging	5	1	5
Delivery	20	1	20
Transportation Insurance (?)	5	1	5
<b>Total Unit Cost, EUR</b>			<b>310</b>

## **Appendix XI: Development expenses**

- a. R&D Expenses: Estimate the costs associated with research, design, prototyping, and testing. Include costs for acquiring necessary equipment and technologies, hiring, and potentially engaging external consultants.
- b. Regulatory Compliance: Factor in the expenses associated with obtaining necessary certifications and approvals for your medical device. May involve engaging with regulatory bodies and compliance consultants.
- c. Intellectual Property (IP) Protection: Include costs related to patent filing and legal fees to protect your innovative technology from infringement.
- d. Manufacturing Setup: Estimate the costs of setting up manufacturing facilities or outsourcing production to third-party manufacturers. Consider equipment, tooling, quality control, and logistics.
- e. Marketing and Distribution: Account for costs associated with marketing, branding, and promoting your product. Additionally, include expenses related to distribution channels, such as establishing partnerships or developing an e-commerce platform.

## Appendix XII: Break-even analysis

Year	Revenue	Total cumulative comparison development costs
0	€ 0,00	-€ 4.700.000,00
1	€ 156.000,00	-€ 4.544.000,00
2	€ 156.000,00	-€ 4.388.000,00
3	€ 156.000,00	-€ 4.232.000,00
4	€ 358.800,00	-€ 3.873.200,00
5	€ 358.800,00	-€ 3.514.400,00
6	€ 412.620,00	-€ 3.101.780,00
7	€ 474.513,00	-€ 2.627.267,00
8	€ 545.689,95	-€ 2.081.577,05
9	€ 627.543,44	-€ 1.454.033,61
10	€ 721.674,96	-€ 732.358,65
11	€ 829.926,20	€ 97.567,55

