Team Results Document
TU Eindhoven Sensing Team

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SensUs

SensUs 2023
Traumatic Brain Injury

1. Abstract

T.E.S.T. has developed a small and user-friendly point-of-care biosensor for rapid traumatic brain injury (TBI) diagnosis for optimal patient recovery. TBI is a neurological disorder which imposes a significant burden on public health, with yearly 85.000 patients in the Netherlands (1) (2).

The biosensor is based on plasmon-enhanced fluorescence using gold nanorods (AuNRs). The assay makes use of anti-GFAP antibodies that are covalently coupled to AuNRs and fluorescent dyes, forming a sandwich complex upon the binding of GFAP. Excitation of a sample by a laser leads to enhanced fluorescence of the dye within the generated electromagnetic field of the AuNRs, contributing to the high sensitivity of the assay. This fluorescence signal is measured in an epifluorescence setup comprised of a built-in microscope system with image analysis capabilities. The measured intensity of the light of individual AuNRs is used to derive the concentration of GFAP in the sample within 10 minutes. With this concentration, the severity of TBI can be rapidly determined, after which the treatment can be started immediately, thereby improving the quality of patient care.

2. Biosensor system and assay

2.1 Overview

T.E.S.T. developed a small point-of-care device to rapidly determine the severity of Traumatic Brain Injury. The biosensor is based on plasmon-enhanced fluorescence. The plasmon enhancement occurs when the fluorophore is in the vicinity of the gold nanorods, as shown in **Figure 1**. The fluorescent signal is measured using a self-built optical system, after which the signal is processed. The result will be displayed on the screen of the biosensor.

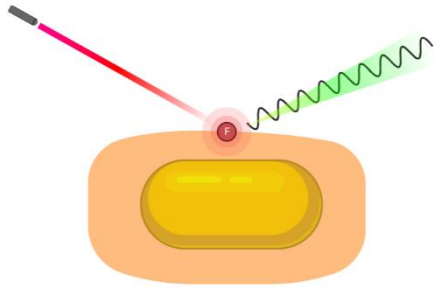

Figure 1: **Schematic overview of the plasmon enhancement**. *The orange area is the enhanced electromagnetic field. The fluorophore is present in the enhancement area and emits a fluorescent signal when excited by an external light source.*

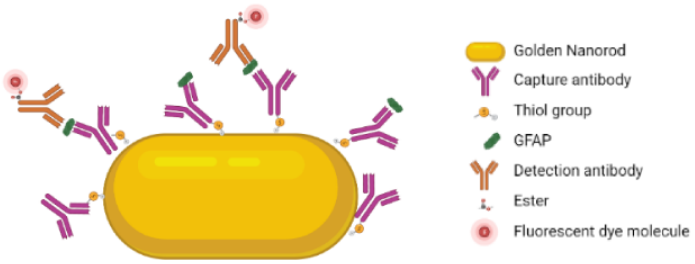

Figure 2: **Schematic overview of the sandwich immunoassay.** *GFAP can bind to capture antibodies immobilized on the AuNRs and, subsequently, a detection antibody can bind to the GFAP. Created in Biorender.com.*

2.2 Molecular recognition and assay reagents

The sensing principle is based on plasmon-enhanced fluorescence, where the intensity of the light on a single-particle level is proportional to the concentration of GFAP. Gold nanorods (AuNRs) are illuminated with a laser with approximately the corresponding plasmon resonance wavelength of the rods (650 nm), which will create localized surface plasmon waves at the surface of the AuNRs (3). The surface plasmon waves create an enhanced electromagnetic field in the vicinity of the particle. If a fluorophore is present in this field, its quantum yield and excitation rate increase, leading to a substantial enhancement of the fluorescence (4).

The assay is a sandwich immunoassay using two monoclonal anti-GFAP antibodies performed directly in human blood plasma. Fluorescently labelled anti-GFAP antibodies (GFAP81cc, HyTest) were obtained by covalently coupling these to NHS-activated ATTO-655 dye molecules (Merck) (**Appendix 1A**). The AuNRs (Au NR-40-650, NanoSeedz) are immobilized onto a glass coverslip (22x40mm, Menzel) by pipetting it on the glass surface functionalized with MPTMS ((3-Mercaptopropyl)trimethoxysilane). The trimethoxysilane group of MPTMS binds to the glass, while the thiol group can bind to the gold of the AuNRs, attaching them to the slide with a semi-covalent bond. Anti-GFAP coupled AuNRs were obtained by first thiolating the capture antibodies (GFAP15cc, HyTest) with Traut's reagent (2-iminothiolane, Merck), which could then form disulfide bonds with the AuNRs. BSA (Merck) was incubated on the glass surface of the cartridge to diminish nonspecific interactions of the dAbs and GFAP. The binding of the detection antibody in the sandwich assay causes the dye to be in proximity to the plasmon-enhanced field. The overview of the sandwich assay can be seen in **Figure 2** above (4).

2.3 Physical transduction

The formed sandwich complex in the assay leads to an enhanced fluorescent signal on the glass substrate. In order to measure this response, an optical setup was built that is able to measure the emitted light of the enhanced dyes, while ignoring light from dyes in the bulk fluid and the excitation light source.
As shown in **Figure 3**, light emitted by a 637 nm laser diode (Thorlabs HL6388MG) is used to excite the nanorods on the sample surface, creating a fluorescence emission response. In the optical path of the setup, the laser light first encounters a dichroic mirror (Edmund optics #67-068) with a cutoff wavelength of 660 nm, where the most optimal reflection wavelengths are between 581-651 nm, positioned under an angle of 45 degrees, reflecting the light in a 90-degree angle towards an objective. The objective (Edmund optics MSB50400) has a numerical aperture of 0.65 and a 40x magnification. Being focused by this objective, the light reaches the sample and is scattered and absorbed by the assay. Both the scattered and emitted light travel back through the objective and reencounter the dichroic mirror, through which the emitted light passes. Most of the laser light is reflected, significantly reducing its share in the remaining signal. The signal then encounters two optical filters: first, a notch filter (Chroma ZET635NF) with cutoff wavelengths of 635 nm, filtering out the

remaining laser light, and subsequently, a high pass filter (Chroma ET655lp) that cuts off at 650 nm to reduce other possible noise present in the signal. The remaining light is captured by a monochromatic camera (FLIR BFS-U3-31S4M-C), forming a fluorescence image. The captured image is then processed by the sensor's software on a Raspberry Pi 4, quantifying the fluorescence intensity, which is correlated to the concentration of detection antibodies and thus to the amount of GFAP that has bound. In order to obtain a clear and useful image, the objective needs to be 0.6 mm (working distance) from the sample slide and 16 cm from the camera. These distances are incorporated into the design of the setup. To place the sample in focus, the objective, which is mounted to a cart that can slide over a rail, can be carefully moved up and down along the z-axis by a linear actuator stage. The linear actuator uses a driver circuit to break down and control each step taken, allowing a precise and reliable 0.4 µm step, corresponding with the objective's 0.4 µm depth of view and therefore providing the required accuracy to focus the images properly.

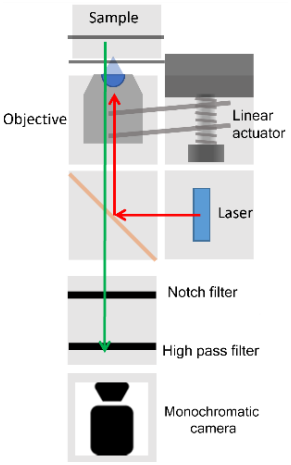

Figure 1: *Schematic optical setup.*

2.4 Cartridge technology

The cartridge is a glass coverslip with an imaging spacer (SecureSeal™ Imaging Spacer -SS8X9, 8-9mm Diameter ID X 0.12mm Depth, 25mm x 51mm, Grace BioLabs) on top. First, the GFAP sample is pipetted outside the biosensor into the imaging spacer. After this, the sample is washed with PBS. Next, the solution of dAbs functionalized with ATTO655 fluorophore is added inside the imaging spacer. After incubation, this is washed with PBS. The cartridge is then ready to be inserted into the biosensor. The total incubation time is 20 min. The procedure described here is used to prove the feasibility of the sensor. A procedure to develop the cartridges for the PoC sensor has yet to be developed, but will be addressed in section 3.3.

2.5 Reader instrument

This biosensor uses a camera for sensing through a grayscale image obtained in the Near-Infrared spectrum. The images taken by the camera are saved onto the Raspberry Pi 4. These images all consist of a black background, bright spots caused by the rods and dye molecules, and some additional background light caused by dye molecules, blood serum proteins, or random noise. An algorithm analyzes each image to determine the concentration of GFAP, which is based on first subtracting this background, then determining the locations of the particles via a threshold, and finally adding up the intensities of the pixels inside the determined particles and averaging them out.

Figure 2*: Biosensor design*

The components are powered by the main voltage from, for example, a wall outlet. The dimensions of the biosensor are 40x20x25 mm. **Figure 5** shows the exterior design. The alignment of all the optical components is achieved by placing them in a custom 3D-printed setup. The laser is placed in a metal thermal mount, through which heat can be dissipated. This slows down the build-up of heat in the laser diode, increasing the stability and reproducibility of measurements. Finally, the casing around the setup is designed to entirely block any light coming in from the surroundings, ensuring that none of the optical measurements are influenced by ambient light.

2.6 User interaction

User-interface and user-friendliness. The sensor has an LCD touchscreen (7-inch TFT-LCD Display 1024x600 pixels with a touchscreen) displaying a graphical user interface (GUI). The GUI was designed to guide the user through the measurement to make it more user-friendly.

Performing a Measurement. For achieving a measurement, several practical handling steps are needed. Step 1: Add the GFAP sample to the glass slide with the immobilized nanorods. Step 2: Wait for 10 minutes to incubate the GFAP. Step 3: Wash the slide thoroughly with PBS. Step 4: Pipet the dAbs solution to the glass slide. Step 5: Wait for 10 minutes to incubate the dAbs. Step 6: Wash the slide thoroughly with PBS. Step 7: Insert the cartridge into the biosensor. Step 8: Focus the camera by using the up and down buttons on the GUI while looking at the camera view on the GUI. Step 9: Take a picture with the save button. The concentration of GFAP in the sample will be calculated from the image and will be displayed on the screen together with the corresponding severity class of TBI.

3. Technological feasibility

3.1 Molecular recognition and cartridge

To functionalize the AuNRs with cAbs, two methods were tested: 1. incubating cAbs on immobilized AuNRs on a glass slide (drop-casting) and 2. incubating cAbs with AuNRs in solution using salt aging (5). The second method led to the clustering of the AuNRs in the solution and a large inter-sample variability in the total number of observed particles (see **Figure 5**). Clustered particles on a cartridge give a higher intensity compared to single particles while still being counted as a single particle during image processing, leading to an unrealistic high intensity per particle in a sample. For these reasons, the first method was chosen for AuNRs functionalization, which led to less particle clustering and a smaller variability in particle density between cartridges.

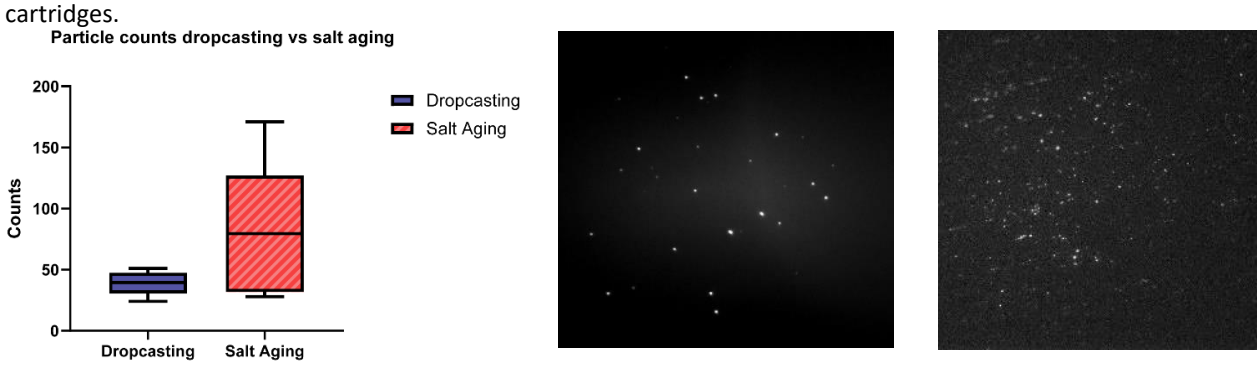

Figure 5: Comparing drop-casting with salt aging. *On the left: boxplot of the particle counts of the AuNRs with drop-casting and salt aging. The counts are in the same field of view size. In the middle: picture of the AuNRs with drop-casting. On the right: picture of the AuNRs with salt aging.*

The sandwich assay was first analyzed in an experimental setup using a confocal microscope (Nikon, Eclipse Ti-E). For a measurement, 50 µL of blood plasma containing GFAP was added to the cartridge. After 10 minutes of incubation, the cartridge was washed with PBS, followed by the addition of 50 µL 1nM dAb. After 10 minutes of incubation, a second washing step was performed. Then a picture was captured via the microscope. The resulting dose-response curve can be seen in **Figure 6**. The assay performs well in both PBS buffer and blood plasma, with a calculated limit of detection of 0.41 pM (= 16 pg/ml) in blood plasma. The analytical performance of the assay measured with the PoC biosensor has yet to be determined.

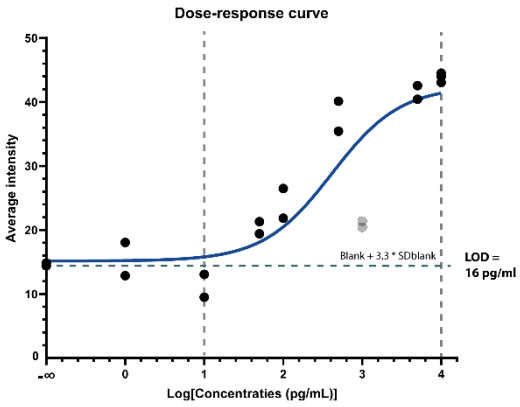

Figure 6*: Dose response curve of the GFAP concentration in blood plasma. The dashed lines represent the relevant clinical concentration range (0.01 – 10 ng/ ml). The image was taken after around 20 minutes of incubation in total. The samples were measured in duplex (2 separate cartridges). A non-linear fit was fitted in GraphPad. The EC50 is 412 pg/ml. The LOD is ≈ 16 pg/ml and is calculated with blank + 3,3*SD$_{blank}$.*

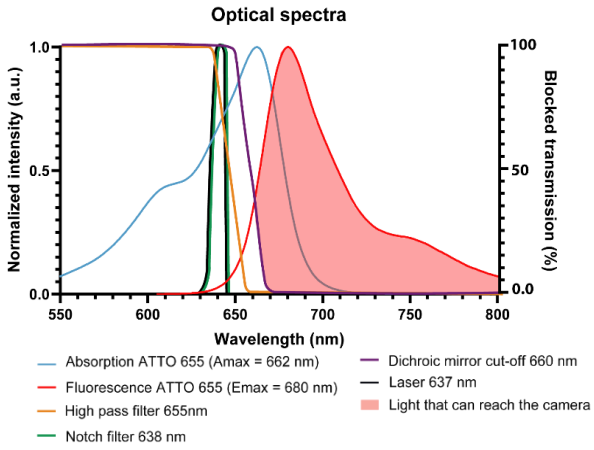

Figure 7*: Overview of the optical spectra. This figure plots the typical absorption spectrum of the dye, the emission spectra of the dye and the laser, and the blocked transmission spectra for all three filters (high-pass, notch and dichroic mirror). The surface highlighted in red depicts how much of the dye's emission spectrum is not blocked by the filters, and thus reaches the camera.*

3.2 Physical transduction and reader instrument

The laser used to excite the dye is 637 nm, and filters are chosen accordingly to reduce the light intensity of the laser in order not to affect the picture created on the camera. The effectivity of the filters in blocking the laser light was confirmed experimentally using a Thorlabs CCS200/m Spectrometer (**Appendix 1B**).

The interaction of the optical components can be represented in a graph displaying the optical spectra of components, as can be viewed in **Figure 7**. The optical density of the filters is 6.55 OD of the Notch Filter and 6.02 OD of the Long-pass filter, meaning that the transmission blocked is nearly 100 percent.

The image captured by the camera is processed in a few steps. First, the image is saved as a grayscale 8-bit .tif file to the Raspberry Pi storage. In order to remove the background, a white top-hat filter with a kernel size of 10x10 pixels is applied (**Figure 8**). Next, this filtered image is converted into a binary mask containing white pixels at the locations of the bright spots, representing the rods and dye molecules (**Figure 8**). This binarization uses a threshold grey value of 15 (determined experimentally to yield the best results). Finally, the original grey values at the locations of the white pixels in the mask are added up and divided by the number of white particles in this mask. The obtained value, the mean light intensity per rod, is used in the calibration curve.

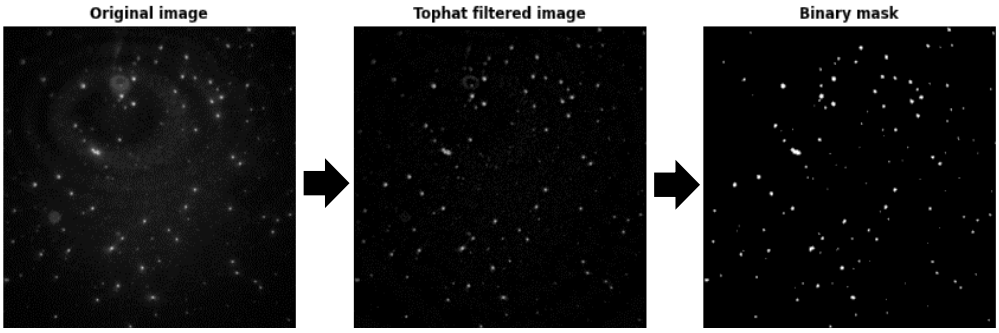

Figure 8: **Overview of the image processing steps**. *First a top-hat filter is applied on the original image, after which a binary mask is made. This mask is applied to the original image, and the mean light intensity per rod is calculated.*

3.3 Future improvements

Currently, the diode is cooled passively by dissipating heat through the laser mount, which effectively slows down the heat buildup. However, active cooling may provide more reliable control over the temperature, thus ensure laser stability even when measuring for extended periods of time. Another option would be to implement an electronic control circuit that monitors the operating temperature of the laser and changes the electric current accordingly, in order to keep the light intensity stable for different temperatures. Furthermore, the laser used in the prototype is not collimated, which means the laser beam diverges from its origin. As a result, the light loses intensity on its way to the sample. When using a collimated laser instead, the same excitation intensity at the sample could be achieved when using a less powerful laser diode. This would reduce heat buildup, improve laser stability, and cut costs.

Since the position of the slide is fixed and the objective can only move along the axis perpendicular to the slide, there is currently no way of intentionally selecting a field of view on the slide surface. By attaching the slide holder to an XY stage, the field of view could be repositioned between slides/measurements to analyze an optimal number of rods at once, making the measurements less dependent on the location of the droplet.

It is important that the cartridge is improved, as currently two handling steps are needed to wash away the GFAP and the dAb. To make the cartridge suitable for POC, it is essential to remove this washing steps. A possible method to implement these washing steps in the cartridge could be done via a microfluidic system, for example similar to the system of Disch et al. (6). The cartridge would have a simple system, where the washing buffers and dAbs can be stored in blisters. When pressed, the fluid comes out of these blisters and into the system. A simple reservoir would catch the waste. Using these cartridges, the user only needs to add the blood sample in the inlet and then press on the blisters to wash. After incubation of the sample, add antibodies and wash again to remove unbound detection antibodies. A different way would be to investigate mixing the blood sample together with the dAbs prior to adding these to the cartridge. This would reduce the number of washing steps within the cartridge and possibly also reduce the total time-to-result. Long-term storage of cartridges could be achieved by using a sugar-based vacuum-drying method to preserve the substrate surface and the antibody-functionalized AuNRs. This has previously been described by Lin et al. (7), where it was found that the cartridges could be stored up to 3 weeks without significantly affecting the sensor activity.

4. Originality

4.1 Team captain

We started exploring several sensing principles for detecting GFAP in literature with the team. The decision to use plasmon-enhanced fluorescence was based on the interests of the team members, since it is a challenging method with a lot of image processing, and on the knowledge available within the molecular biosensing (MBx) research group of TU/e (8). Our initial idea was inspired by an article that used gold nanopyramids, but the concept changed to rods after it became clear that producing these particles ourselves was unfeasible (9).
The principle of the sandwich assay was based on an assay method used in the MBx group. In the group, researchers work with gold nanorods with immobilized DNA strands. The team adapted this method to a sandwich immunoassay that utilizes anti-GFAP antibodies that has been reported before in literature (10). The usage of Traut's reagent for thiolation of the antibodies was found based on Wang et al., 2017 (11). The functionalization of the nanorods is based on protocols from Vincenzo Lamberti (MBx at TU/e).
For the physical transduction, the wavelengths of the plasmon wave of the gold nanorods, the laser, and the fluorophores needed to be carefully selected. Many different combinations have been considered, and the final combination was selected based on research in the MBx group. The electrical circuits for the Raspberry Pi, the LCD touchscreen, the laser, the linear actuator and the camera were all designed and assembled by the team itself. The design of the optical setup was based on general microscopy configurations and the design of an existing setup available within the MBx group. The design was adapted for the biosensor application and built from scratch with custom 3D-printed components of our own design. Furthermore, a unique combination of filters has been created and tested to completely block out the light of the laser, whilst getting as much signal as possible in the right wavelength window. The software involved in the sensor, including the image processing, the software for the linear actuator stage and the GUI, is written by ourselves. Finally, we individually designed and built the final biosensor, as a team.

4.2 Team's supervisor

The team started with a literature search and came up with a shortlist of two sensing principles: impedance spectroscopy and the use of gold nanopyramids to enhance the fluorescence yield in a sandwich assay. After consulting Palmsense, impedance spectroscopy was discarded, and the idea was explored to deposit ordered nanopyramids using colloidal particles and electrochemistry. The team used a considerable amount of time in this project phase and was unsure how to proceed. The supervisors confronted the team with the need to start working in the lab, and the team finally decided to use commercially available gold nanorods after consulting researchers in the MBx group at TU/e. The team developed their own strategy to couple antibodies to the AuNR based on literature and has been supported by researchers in the group MBx to investigate and demonstrate the feasibility of the assay on a large commercial microscope in the laboratory. The construction of a "miniaturized microscope" was inspired by setups available in the MBx group, but the team decided to build an optical system from scratch. After a failure using cubic building blocks available at TU/e, they decided to design and manufacture a setup completely by themselves using a 3D printer to incorporate the required components. The team showed a strong and admirable incentive to build their own system, which however backfired due to the required complexity and required amount of time which was underestimated. The final weeks of the project will show whether their own integrated miniaturized microscope will work successfully.

Signatures

L.J. (Leo) van Ijzendoorn	**S.J. (Sterre) de Lignie**	**S.R. (Sam) Reijs**
Team supervisor	*Team captain*	*Team captain*

5. Translation potential

5.1 Business model canvas

<table>
<tr>
<td rowspan="2">Key Partners
Institutions
- Hospitals
- Health insurance companies
- GGD Nederland
Research and government
- University of Technology Eindhoven
Production partners
- Antibodies-online
- NanoSeedz
- Chroma
Technical expertise
- Neways
- Alten BV
- ASML
Practical knowledge
- Neurologists
- General practitioners</td>
<td rowspan="2">Key Activities
- Research, development and optimization of an accurate, and rapid point-of-care biosensor to say ahead of the curve
- Competing in SensUs competition with working biosensor including biorecognition and detection method
- Getting Intellectual property rights and maintaining them
- Hold close contact with end-users and partners
- The production of the biosensor and its cartridges
- Achieve stable market position and research expansion into new markets

Key Resources
- Dedicated team consisting of 8 people with different backgrounds
- Knowledge of neurologists and General practitioners
- The desire for POCT keeps increasing, this will also lead to more demand for the sensor
- Funding and technical expertise from the partners
- Efficient, accurate and fast point-of-care biosensor
- Laboratory and microscope lab facilities</td>
<td rowspan="2">Value proposition
- The biosensor will be an improved version of current diagnostic methods. It will be more cost-effective, and more accurate than current assessments of Traumatic Brain Injury (questionnaire, CT, MRI scans).
- The point-of-care device will reduce specialist occupation as it is more user friendly and faster than CT-scans operated by specialists. The diagnosis can be given within 10 minutes, which also relieves work pressure.
- Patients will receive a correct and reliable diagnosis, where before a correct diagnosis could not be provided.</td>
<td>Customer Relationship
- Less time for customers as appointment time decreases
- Constant contact with doctors on the performance of the biosensor and feedback on quality of life improvements
- High reachability to come into contact with the team with problems for existing and future customers.

Channels
- Biomedical, electrical, business and computer science departments of the TU/e for expertise and future highly-educated employees
- University based hospitals for general feedback on device including GP, neurologists and doctors.
- Online platform and social media contacts.</td>
<td>Customer segments
- Hospitals
- Insurance Companies
- Doctors
- Neurologists
- Patients
- GGD Nederland</td>
</tr>
<tr>
<td colspan="2">Cost structure
Variable costs:
- Production costs per device (€550 per device)
- Production costs per cartridge (€0,20 per cartridge)
- Marketing
- Personnel
Fixed costs:
- Research & development
- Rent
- Medical license + clinical trials
- Quality assurance
- Intellectual Propery costs (patent, tm, etc.)</td>
<td colspan="2">Revenue Stream
- Device sales (€1500,- per device)
- Cartridge sales (€30,- per cartridge)
- Maintenance
- Funding & grants
- Investments</td>
</tr>
</table>

Table 1*: Business Model Canvas T.E.S.T. 2023*

5.2 Stakeholder desirability

Traumatic brain injury (TBI) is a neurological disorder with the highest incidence rate compared to other common neurological conditions, and imposes a significant burden on public health (1). It can be caused by a vigorous impact, strike, or sudden shake to the head or body, or by an object penetrating the skull and reaching into the brain (12). It is increasingly recognized as a chronic disease with long-term consequences that may increase the risk of developing late-onset neurodegenerative conditions (13). It is estimated that yearly 78.200 people get traumatic brain injury in the Netherlands (14). On average, yearly, 47.100 people visit the emergency room of the hospital with brain injuries caused by accidents or violence.
Currently, Traumatic Brain Injury (TBI) is diagnosed using two primary tools. Firstly, by questionnaires and the Glasgow Coma Scale, which is not quantitative and is inadequate when the symptoms are unclear or when

there is overlap with other issues like depression, the influence of alcohol at the time of the accident or traumatic events (15) (16). Secondly, in moderate and severe cases, often a CT scan is performed to assess the severity. However, CT scans are expensive, as the average cost of a CT scan of the brain is around 1270 euro (17). Even more, CT scans do not always show signs of damage in patients that do have TBI, since 90–95% of scanned patients do not show intracranial injury, while these patients are subjected to the risks of radiation (1). This radiation increases the risk for radiation-induced carcinogenesis (18). MRI scans can also be used, but they are even more expensive and the measurement takes long (19). Because of the high costs, many people do not receive the CT scan which can help with the verification of TBI, and it is difficult to determine who needs to receive such as scan. As there is no other reliable method to diagnose TBI, many patients will not get the correct diagnosis. Therefore, a new method to diagnose traumatic brain injury is needed.

It has been shown in International Traumatic Brain Injury Research studies that the measurement of biomarkers in the blood can add value to clinical decision rules used for diagnosis and for the decision about making a CT scan or not (20). The detection of biomarkers can improve efficiency and additionally decrease the number of patients exposed to radiation. At the moment, there is only one company on the market, Banyan Biomarkers, that produces assay kits that measure biomarkers for TBI (21). This assay kit can be used as an adjunct to standard clinical practice to aid in the evaluation of patients with a head injury. This test is not yet on the market and needs to be used by professionals in a clinical laboratory setting and uses multiple devices (22). Another alternative is a method that involves eye-tracking procedures, but this method focuses mainly on sport-related concussions (23). When a traumatic brain injury is not properly diagnosed or not diagnosed in time, this can lead to irreversible brain damage, and patients who receive immediate medical care often have the best health outcomes (12). Therefore, a new method to assess the degree of TBI is needed that can be performed easily by a health specialist without many specialized and expensive devices (24). Consequently, a point-of-care biosensor that can measure the concentration of clinically relevant biomarkers to help diagnose TBI is highly needed. It has recently been established that blood GFAP levels are correlated with clinical severity, so this biomarker is the target for T.E.S.T.'s biosensor NeuroTEST (25). The biosensor is less expensive, more accurate, and quick in diagnosis, with results within 10 minutes, compared to methods currently used for diagnosis. In addition, it is portable and user-friendly. Availability of such rapid blood tests for TBI will help healthcare professionals determine the severity of the TBI and the possible need for a CT scan in patients suspected of intracranial injuries. This will also help prevent unnecessary neuroimaging and associated radiation exposure to patients. Furthermore, more patients will receive the correct diagnosis as more patients can be tested with this biosensor since it is low-threshold use. It will also decrease hospital workload by reducing the number of CT scans needed. The mission of T.E.S.T. can be summarized as follows: *Rapid diagnosis of Traumatic Brain Injury on the spot for optimal patient recovery.*

When looking at several options where the biosensor could be implemented, such as ambulances, general practitioners (GP), sport's medical staff, there were three customer segments deemed most viable, namely emergency rooms (ER), general practitioners and neurologist offices, which were analyzed (**Appendix 2**). The market where the sensor's use would be most optimal should abide by certain characteristics, such that the sensor is operated by users that can take blood samples and that there is a stable supply of people needing to be tested. After multiple interviews with medical professionals and literature research, it was concluded that NeuroTEST is the most attractive for usage in the ER (**Appendix 3**) (26) (27) (28) (29). Here, overcrowding and prolonged waiting time threaten the quality of patient care, and point-of-care devices could help significantly to reduce overcrowding (30). Moreover, patients who have fallen on their heads, for example, will first go to the ER.

Interest in a PoC biosensor such as NeuroTEST can be found at several different stakeholders. First and foremost, the patient who experiences TBI. By accurate and quick measurement of GFAP concentrations, the correct diagnosis can be determined shortly after the injury, and the optimal treatment plan can be started almost immediately, providing the patient with the care they need to recover as much and fast as possible. Secondly, doctors and health personnel are stakeholders because they profit from this new technique by decreasing the diagnosis time and increasing the number of patients that can be diagnosed without referencing them to for instance a neurologist. The decrease in unnecessary workload on the neurology department and the quick diagnosis could lead doctors and health professionals to convince hospital procurement to purchase the biosensor. Thirdly, hospitals themselves are a stakeholder since they will buy the sensor. Moreover, for hospitals, it is important if a product cost can be covered by the Diagnose Behandel Combinatie (DBC, diagnosis treatment combination) compensation of the health insurance (31). A DBC consists

of all the costs from when a patient enters a hospital until their treatment is done, with a maximum of 120 days (32). Therefore, a cheaper and quicker diagnosis would save costs for this, which would also benefit the health insurance companies. Lastly, the health insurance companies are an essential stakeholder, for whom it is most important to reduce patient care costs. With NeuroTEST, there will be a significant reduction in CT scans and prolonged treatment trajectories. This reduction of costs will in turn lead to insurance companies encouraging the hospitals to obtain the biosensor (**Appendix 4**).
The procedures a patient goes through with the current clinical practices are illustrated in a customer journey (**Appendix 5a**) based on clinical standards and the experiences of TBI patient Niels de Laat (28). The impact of the biosensor on the patient is illustrated in a second customer journey (**Appendix 5b**). Obviously, NeuroTEST has an enormous impact on the diagnosis process. At the ER a finger prick will be performed, whereafter the result will be visible within 10 minutes, and the treatment plan can be formed.

5.3 Business feasibility

Key resources

The main priority until at least 2026 will be continuous research and development by the Eindhoven University of Technology team, where the focus will lie on improving the sensor for more accurate, faster and user-friendly point-of-care diagnoses. Moreover, the cartridge handling will be improved via an ameliorated microfluidic chip to reduce the handling steps. In order to grow, it is needed to expand this research and development team into a new location where laboratories and microscopy facilities are available to support future scientific endeavours.

In addition, after the clinical trials have started, stable contact with the users is essential, such as with the health professionals at the ER, who can provide information on how the sensor is operating. This feedback will aid in improving the biosensor to be even more user-friendly and convenient for those designated to operate the device. To ensure that this is feasible, a separate team of account managers will be created who will uphold relationships with users and valuable assets of information. Moreover, a business department, ICT and human resources will be necessary to create a functioning company structure. In order to acquire all these resources and expertise, the support of partners is required that will accelerate growth via funding and knowledge. In **Appendix 6** and **Appendix 7**, a SWOT- analysis and a MoSCoW analysis have been added,respectively which give an overview of the team's strengths and weaknesses, and help with the overview of the current resources.

Key activities

To achieve successful commercialization, the customer-centered RACE marketing model will be used to effectively engage with the customers, which is a classic marketing funnel. It uses the iteration of Plan, Reach, Act, Convert, and Engage to build a long-term thriving organization connected to its customers. First of all, it is crucial to reach out to Key Opinion Leaders (KOLs), doctors and researchers with a prominent position in the field of TBI who will be asked to help by doing clinical research on the product and presenting it to others. This way, It is possible to reach other doctors who can purchase a biosensor for their own hospital (31). To find these doctors, contact with the NFU (Nederlandse Federatie van Universitaire Medische Centra) can be made or visit one of the events where these doctors are present to get in contact with researchers. Furthermore, talks with and relationships with hospitals close to Eindhoven, where the team is based, will be made. Moreover, the aim is to promote the use of the biosensor via health insurance companies as well as via the European Ministry of Health, through medical federations, and patient organizations. With compelling results from the patient trials and R&D, an effort to convince organizations of the added value of NeuroTEST can be made.

The core tasks of the team are the development and optimization of an accurate, and rapid biosensor. Continuous research will be performed to make it more rapid, more sensitive, and to make it compatible with future multiplex detection, because the use of the biosensor will be expanded to a future multiplexing device to make the biosensor even more specific for Traumatic Brain Injury via the measurement of other biomarkers for TBI, such as S100B (33). Next to this, the production of the sensor and its production planning are essential. Moreover, finding collaborations of third parties are a priority of T.E.S.T. to raise funds, gain additional

knowledge, and partner up for materials. Lastly, market research is vital to optimize the product-market fit and expand into new markets outside the Netherlands.

To become the exclusive manufacturer of the newly developed biosensor and to protect the market share, it will be necessary to acquire licenses and patents for different aspects of the sensor (34). Furthermore an authorized representative needs to ensure that the biosensor upholds EU standards and upkeep CE markings to ensure a legal and fair trade for the manufacturer. This ranges from the implemented microscopy setup to the preparation and creation of the cartridges, which are to be implemented into the sensor. Currently, Vincenzo Lamberti is also in the process of acquiring a patent in the plasmon-enhanced fluorescence field, so for the sensor principle, it is necessary to make sure the patents are complacent and solely acquired by the team with that in addition to other patents to might have already been filed in this field to account for other patents which might already be filed in this field. The process to acquire these patents will be started early on during the R&D phase, and it will be determined if it will be handled internally by the team or outsourced to a specialized company.

Clinical studies will be required to prove the proposed benefits that emerged in the interviews with several medical specialists and general practitioners in the medical field (26). This will be conducted in collaboration with ERs throughout the country and with hospitals near Eindhoven and will be performed from 2026 till 2028 (see timeline in **Appendix 8**). In addition, it will be necessary to provide certain quality assurance to ensure the reliability and principles of the sensor. In order to manage this, EMA approval is required after successful clinical trials where CE certificates can be acquired.

During these medical trials, upscaling of the sensor and cartridge production will be researched. The implementation of products produced by original manufacturers will also be essential in this process to reduce the costs of the biosensor. Injection moulding is also a viable option for reducing the costs when upscaling the production of the larger components in the biosensor and for components of the cartridge (28) (35) (27) (29). The goal is to enter the market in 2029 in the Netherlands at ERs. Once the product has been implemented in the ERs, the market strategy will expand to hospitals without an ER, where it can be used at the neurology department. Next, the aim is to launch the biosensor in 2031 in a small portion of European countries (Belgium, Germany, and France) to adapt to problems that arise in other countries before fully launching in Europe in 2035. Furthermore, the plasmon-enhanced gold nanorod technology combined with the sandwich assay has great potential to become a platform that can be used to diagnose a large variety of diseases, using antibodies specific for different biomarkers (**Appendix 9**). This way, more diverse cartridges can be developed.

Key partners

The key partner of T.E.S.T. will be health insurance companies that will reimburse the product for hospital usage. For the production, T.E.S.T. relies on antibodies-online to deliver of the required antibodies with high specificity, NanoSeedz for the nanorods on the cartridge, and Chroma for the distribution of the essential filters for the biosensing device. For the technical expertise, T.E.S.T. relies on the key partners from Neways Electronics, Alten BV, and ASML to aid with the electronics setup within the device and advise on the core building blocks upon which the business plan will be set. Maintaining close contact with the partners will ensure that the respective wishes of both parties will remain in sight and the plans can be aligned accordingly with these wishes. This will ensure a mutually beneficial collaboration with all parties.

Sustainability

To minimize the environmental impact of the biosensor, the plan for the future is that hospitals can hand in their current sensor whenever a newer version is released and they want to replace the previous model. If they choose to hand in the older device they get a discount when buying a new biosensor. The performance of older devices and their components will be checked, and based on the results the devices can either be sold second-hand to hospitals that do not want to pay a premium price for the latest device, or the components can be used in the production of new sensors. As for the cartridges, research will be done to find out if the cartridges can be recycled or if they can be used multiple times after a thorough cleaning process. Also, via a sugar coating the cartridges can be stored longer, reducing the amount of waste of expired cartridges and allowing packages in which hospitals can buy cartridges in large batches. This will reduce transport pollution and small individual packaging plastics. Moreover, a look into more sustainable materials will be taken for the

components to produce the biosensor, whilst producing as least waste as possible. Lastly, the goal for the future is to go to a zero CO2 emission process, but still a lot of research will be needed to make this possible. For this, researchers on the R&D team will also focus on this, by for example focusing on more efficient production processes, and by shifting from depletable fossil resources to renewable ones (36).

5.4 Financial viability

The current manufacturing cost of the cartridge is €0.41 (**Appendix 10**). It is expected that these costs can be lowered through research into different production methods, via cooperation with original manufacturers, and by optimizing the assay. Therefore, the final production costs of the cartridge are expected to be around €0.20. The final costs of the prototype biosensor are €2921.79 (**Appendix 11**). For the final sensor, production costs could be reduced with injection moulding, which is a viable option for reducing costs and upscaling cartridges' production (35). Moreover, via cooperation with original manufacturers, it is estimated that the costs of the final sensor will be 20% of the total costs of the prototype, which will result in a price of around €470,-. For the labour costs, it is expected that they will be around 10% of the raw production materials. Moreover, additional ICT, marketing, and distribution costs will be included in the sensor, which are expected to be 6% of costs per device. This results in the final production costs of €550,- (**Appendix 12**).

Hospitals, private healthcare facilities, and military facilities can purchase the device for €1500,-. Per cartridge the cost will be €30,-. Maintenance needed for the device depends on a fixed hourly pay. These prices are based on the predicted costs of bringing the product to the market. The subsequent distribution of devices and tests will be the buyer's responsibility. For the development of the biosensor, a significant amount of research & development will be needed for a final design made with components acquired from original manufacturers and via injection moulding. The costs for these expenses will be around 300k in the first years, and will reduce after the market launch in the Netherlands. However, expenses will continue to be made to develop and improve the biosensor. Next, costs will be made to perform medical trials, which allows application for medical licenses, amounting to around €137.400,- spread over multiple years (37). Also, costs will be made to acquire the needed Intellectual Property certificates (patent, TM, etc.). The cost for a patent application in the EU is around €25 000. After acquiring the patent, yearly maintenance fees for each country are needed to maintain the patent. Therefore, the costs are expected to rise as the years go by. Moreover, costs will be made for renting an office and lab facilities, quality assurance, salaries, marketing, and of course, the production of the devices and the cartridges. (38) (39)

For the revenues, in the R&D and clinical trial phases of the biosensor, the team relies on funding, grants, and investments. After the market launch, revenue is generated via device sales, cartridge sales, and maintenance (see quantities in **Appendix 13**). With current estimations of costs and revenues, it is expected that T.E.S.T. will break-even and become profitable in 2031. A total overview of all the costs and revenues is visible in **Appendix 14** and **Appendix 15**.

Market penetration in the Dutch market is the initial go-to commercial strategy during the proof-of-concept phase. It is expected that as the awareness of TBI grows and the use of PoC diagnostics becomes more prominent, the market for the sensor will grow, which is expected to result in a rising number of sensors and cartridges to be sold. In 2016 the overall healthcare costs among TBI MarketScan enrollees was 40.6 billion US dollars. Low-severity TBI costs, which is most likely a practical use case for the sensor, for the first year after injury, were substantially higher than costs for middle and high-severity TBIs among those with private health insurance and Medicaid (40). For the Netherlands, which will be the initial go-to-market, the costs for TBI in 2010-2012 were 314,6 million euros (41). As these numbers are fairly old, nowadays the costs of treating TBI will have increased significantly. In addition to this, insurance companies always seek the best and most cost-effective way of treatment and recovery. This will lead to them choosing the most optimal, cheapest and fastest recovery time, via the continuous use of the biosensor. The recovery time and CT-scan costs will therefore outweigh the costs of using a biosensor to determine the recovery process. By integrating business intelligence tools and analytics platforms, market trends and sales performance, customer behaviour will be monitored and analyzed to evaluate the effectiveness of the marketing campaigns. This data-driven approach will enable a quick response to changing market conditions, identify growth opportunities, refine product offerings, and ensure that additional value is provided to both healthcare professionals and patients (42).

6. Team and support

6.1 Contribution of the team members

For the development of the biosensor, the team was divided into two sub-teams: the assay team and the detection team. The assay team was responsible for the development of the biochemical assay and the detection team for the physical transduction and reader instrument of the biosensor. Furthermore, the team had a Translational Potential sub-team which worked on the business model of the biosensor. Each sub-team had their own head of the team to keep track of the progress and co-operation within the team. Next to the sub-teams, each member had an organizational function: team co-captain, secretary, treasurer, external relations and public relations.

Team member	Function within the team
Arthur Brim	Head of detection team and Public Relations
Esther van den Bulk	Member of assay team, External Relations and Translational Potential
Merel Diender	Member of detection team, Treasurer and Translational Potential
Lisa van Dooren	Head of assay team, Public Relations and Translational Potential
Tristan van Gool	Member of detection team, External Relations and Translational Potential
Mirte Kleefsman	Member of assay team and Secretary
Sterre de Lignie	Member of assay team, Co-captain and Head of Translational Potential
Sam Reijs	Member of detection team and Co-captain

6.2 People who have given support

During this project, our team has received the help of many people, whose knowledge and advice we greatly value. Our supervisor dr. **Leo van IJzendoorn** advised the team throughout the whole process during weekly meetings. Ir. **Chris Vu** provided support during the weekly meetings and assisted the assay team with writing the protocols. Ing. **Claudia Schot** provided support during the weekly meetings, and was our contact person for lab-related questions and ordering of chemicals or components. The critical questions, the discussions, and the advice we got from the meetings were an enormous help to our team, and we could not have done this without the time and energy you put into this team.
The assay team was advised, and supported by multiples members of the Molecular Biosensing research group (MBx) at TU/e: **Vincenzo Lamberti**, **Koen Valk**, **Yuyang Wang**, **Sebastian Cajigas Bastidas**, and **Peter Zijlstra**. The detection team was aided by **Vincenzo Lamberti**, **Stijn Haenen**, **Sjoerd Nooteboom**, **Lorenzo Albertazzi**, **Marrit Tholen**, **Willem Rovers** and **Martine Duif**. Moreover, we want to thank the **MBx group** as a whole for the access to the laboratories, machines and materials, and for always being friendly and willing to answer our questions.
For Translational Potential, the team was helped by **Bart van Grevenhof**, **Gert Guri** and **Maurits Overmans**. Furthermore, **Annelies Bobelyn** gave the team feedback through feedback sessions of SensUs. The **PowerCompany** helped with the advice and performed an interview to strengthen our business plan. Moreover, the interview with **Micronit** helped with thinking of possibilities to implement microfluidics in the biosensor.

Willem Rovers provided a room to work during the summer, and to do experiments with the optical setup. Eindhoven Artificial Intelligence Systems Institute (EAISI) at the TU/e provided us with a workspace with other motivated teams.

Moreover, with the TU contest, the team won a prize from **ASML**, providing financial support and advice. Next to this, we want to thank all companies and audience at the TU Contest that talked to us and helped by asking questions, giving suggestions, and provided feedback about our idea.

We want to thank **Niels de Laat** for his interview, which helped us with gain a better understanding of living with TBI. **Yasmin Bilderbeek**, **Marc de Lignie, Reinier Wouters**, and **Isa Haeck** for filling in the questionnaire about their symptoms and behavior after a brain injury (**Appendix 16**). In addition, multiple medical specialists helped us gain insight into Traumatic Brain Injury in the clinic by doing interviews with us: **Kim Santegoets**

(Revalidation doctor at *Libra Revalidatie & Audiologie*), **Jikke-Mien Niermeijer** ((children)neurologist at *ETZ Tilburg)*, **Frederique Ummels** (GP working at *Werkwinkel UMCU Utrecht*), and **Sandrina Plas** (GP at *Huisartsenpraktijk Hogeveen en Verhoef*). Furthermore, we want to thank **Arjen Kleefsman** for helping us with the new website after the old one got hacked, due to a lack of updates and a historical version of php, and with making the team pictures.

6.3 Sponsors

T.E.S.T. 2023 would like to show its gratitude for the partners of this year whose support has helped and made this project possible.

Partner	Kind of Support
TU/e Innovation Space	Financial support
Alten BV	Financial support and in-kind contribution (Technical advice)
Merck	In-kind contribution (Funds for their products)
Neways	Financial support and in-kind contribution (Technical advice)
NanoSeedz	In-kind contribution (Gold nanorods)
Chroma	In-kind contribution (Optical components)
Antibodies-Online	In-kind contribution (Anti-IgG)
Hytest	In-kind contribution (GFAP, SensUs Partner)

7. Final Remarks

First of all, it is strange to imagine that our time as a team is almost finished now. Our team is always full of ideas of activities to do together, and everyone is always motivated to put in the time and effort to meet, to participate in events, and to work on the sensor, so we are going to miss this. Being part of T.E.S.T. has allowed us to grow as scientists and to learn many new skills. We learned that it is immensely difficult to translate a theoretical idea into a real-life device, and that many problems only arise after you have started with building and experimenting. Learning how to tackle these problems with a team, and methods how to work more systematically are things we will take with us into the future. But above all, we have had plenty of fun this past year developing our biosensor.

We believe in the considerable value of our biosensor, so in the future we hope our idea and prototype can be completely developed, optimized, verified and validated to finally bring this product to the market (see **Appendix 17**). This can be done by improving the assay via more experiments and by creating a microfluidic cartridge to reduce the amount of handling steps. The sensing device itself has also still many opportunities to be improved, such as the mechanical focusing system. With this improvements, this sensor can be of great help at ERs. In addition, in the future other locations could be explored as well, such as neurologists offices in other hospitals without an ER, or for example the military where many head injuries occur.

Furthermore, the people and companies who supported us were of significant importance for this team. For this, we would like to give thanks to everyone who supported us, especially our supervisor Leo van Ijzendoorn, and coaches Chris Vu and Claudia Schot. Next to this, we thank the friendly and knowledgeable researchers within the MBx group, and the companies whose support and enthusiasm was of great aid to us. Lastly, we are thankful for the energy and dedication of every individual team member, and for the team as a whole.

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9. Appendix

Appendix 1A Calculation of the degree of labeling of the dAbs

For the dAbs labelled with dye, the degree of labelling (DoL) can be determined. To determine the absorption of the antibodies and the absorption of the dye, an absorption measurement is done with a Nanodrop (NanoDrop 2000). The degree of labeling can be calculated with the following formula:

$$\text{DoL} = \frac{A_{max} * \varepsilon_{protein}}{(A_{280} - (A_{max} * CF_{280})) * \varepsilon_{max}}$$

- A_{max} = Absorption of the dye. The average can be calculated from the NanoDrop measurements.
- $\varepsilon_{protein}$ = The extinction coefficient of the protein at 280 nm: 210000
- A_{280} = Absorbance at 280 nm. This is the absorbance from the protein. The average can be calculated from the Nanordrop.
- CF_{280} = Correction factor used in the determination of degree of labeling (DOL) in case of dye-protein conjugates: 0.08
- ε_{max} = The extinction coefficient of the dye at the absorption maximum: $1.25*10^5$

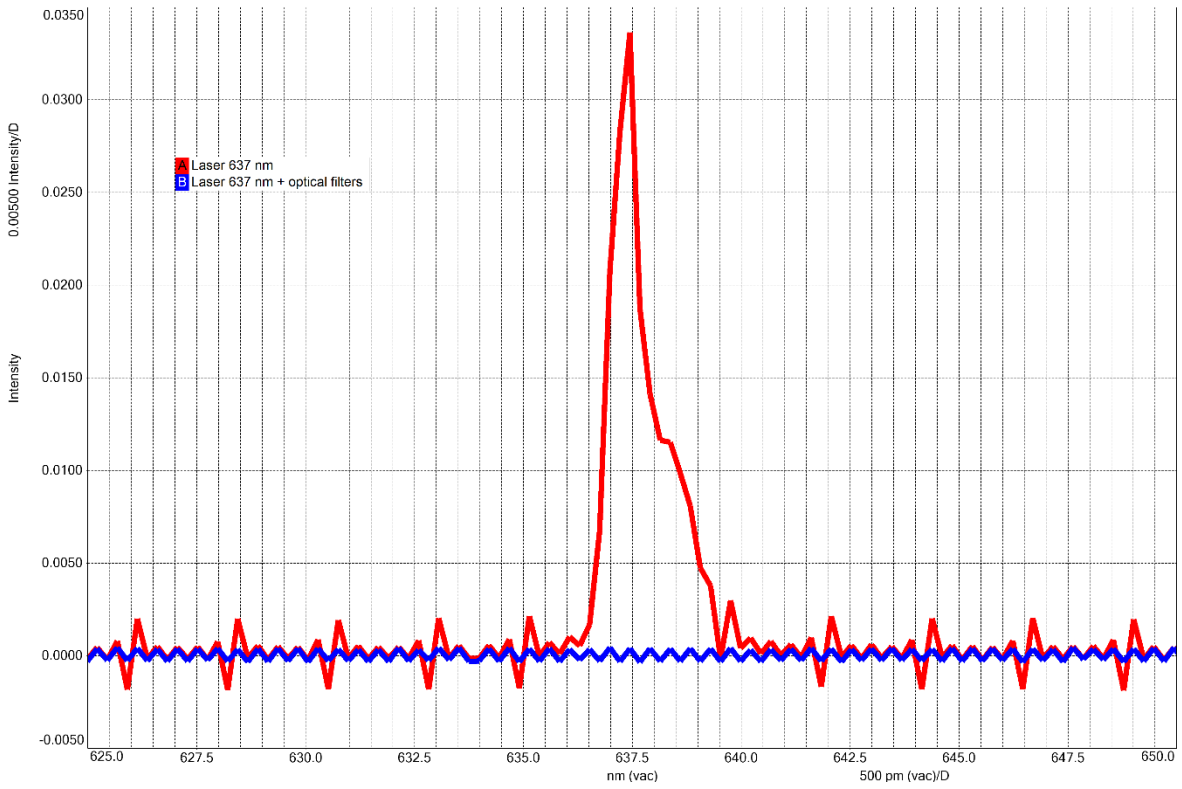
Measurement	A_{280}	A_{max}
1	2.825	1.470
2	2.785	1.455
3	2.970	1.533
Average	**2.860**	**1.486**

$$\text{DoL} = \frac{1.486 * 210\,000}{(2.860 - (1.486 * 0.08)) * 125\,000} = 0.91$$

The degree of labelling of the antibodies with dye is 0.91. This means that there is an average of 0.91 dye molecules per antibody. This Implies that not every antibody has a dye molecule bound to it.

Appendix 1B Spectrum analysis of laser vs. filtered laser

Wavelength spectrum analysis using the Thorlabs CCS200/M spectrometer. The image below was acquired using the compatible software ThorSpectra. As can be seen, the light emitted by the laser diode is almost completely attenuated by the array of optical filters (dichroic mirror + notch filter + high pass filter).



Appendix 2 Customer analysis

Customer segment	Compelling reason to buy	Market volume	Economic viability
General Practitioners	It is an effective way of diagnosing traumatic brain injury in people without having to increase the queue time for hospital scans	Fine: The sensor can be convenient in each general practitioners office if the machine is user friendly and if the result of the test is clear	If included in the protocol for testing injury's concerning the head, it might become a reliable asset. Would be affordable
Neurologist	It can be beneficial to diagnose TBI early on in patients. This could lead to shorter waiting time and more rapid diagnosis	Large: the sensor could be used in many hospitals.	Viable only if the insurance companies are willing to pay, which will allow devices to be installed
First aid at hospitals	Could be useful to early on address the severity of head trauma during an injury	Large: many patients with a head injury first visit the ER	Viable only if insurance companies are willing to pay. In hospitals, ER would be the most logical place

Table 2: *Evaluation of market potential per customer segment*

Customer segment	Implementation difficulties	Time to revenue	External risks
General Practitioners	As the biomarker is not only an indication for TBI, it might be difficult for GP's to assess if the added value of GFAP is related to head trauma	Market would be ready for this solution as it can be implemented as a simple test in the GP office	GPs are already having trouble assessing whether someone has a TBI and might be hesitant to quickly use the sensor for minor cases concerning the head.
Neurologist	Changes in hospitals take time. Also the specialist need to be able to operate the biosensor and implement it in their protocols for the diagnosis of TBI	Market would be ready for the solution, and it can be implemented as a simple test for the neurologist	The neurologist must be able to make a good decision based on a certain concentration. This needs to have high selectively and sensitively in order for specialist to really use the sensor
First aid at hospitals	Implementation of the biosensor should be such a simplicity that anyone with a basic first aid training would be able to operate the device.	Implementation will not take an absurd amount of time, but will be more difficult to convince the public of the necessity of such a device.	Doctors can be conservative, so they need to be sure of the reliability of the sensor

Table 3*: Evaluation of market challenges per customer segment*

Appendix 3 Interview findings

Kim Santegoets, a rehabilitation doctor who specializes in children and young people with head trauma, told us that the diagnosis of TBI is normally done by a neurologist at the Emergency Room in the hospital (29). She believed that a biosensor would especially be useful for patients in the 'in-between group' between moderate and severe TBI who do not show irregularities on a CT or MRI scan. Most of the patients of this 'in-between group' will presumably go to the ER after their head trauma. Smaller accidents, such as a case of bumping your head into a cupboard are seen at the GP's practice. Therefore, we have also interviewed the GPs Sandrina Plas and Frederique Ummels, who indicated that there are several problems with TBI diagnosis at the GP's practice (26) (27). Firstly, estimating the severity of the trauma can be hard. Ummels notes that TBI is often underdiagnosed if she compares the incidence of TBI to the amount of referrals of patients to the hospital for TBI diagnosis. She also says that GPs often do not recognize TBI in time, because the symptoms can be difficult to recognize, as the symptoms for example can resemble a burnout. According to Plas, the most common procedure for diagnosing TBI is through a flow chart. There is one for patients under 16 years old and one for patients over 16 years old. A biosensor could help GPs since a lot of patients have to be sent to the hospital to be diagnosed via CT, while only a few of them actually have TBI. Plas yearly receives around 20 patients with possible TBI in her practice, while around 1 or 2 of these actually gets diagnosed with TBI. The interview with Niels de Laat showed us the impact on someone's life of TBI (28). There really is a life before the brain injury and after, because drastic changes have to be done to one's life and it will be never the same. He stressed the importance of a method to quickly diagnose TBI, since the current methods are extremely insufficient.

Appendix 4 Money saved in the health care system in the Netherlands

In a study in the Netherlands in 2012, 34% of patients at the ER with mild TBI received a CT scan (43). Assuming this percentage is approximately the same in 2023, as more recent numbers are not published for the Netherlands, the following calculations can be made (using 47.100 which is the number of annual patients in the Netherlands (14) and 1270 euros which is the average price of a CT-scan (17)).

- 0,34*47.100 = 16.014
- 16.014 * €1270= €20.337.780

With the biosensor, it is expected that a bit more tests will be performed, since the threshold to perform the test is lower than performing a CT scan. With a selling price of €1500,- per device and a price per test of €30,-, and a 50% increase in the number of tests (16.104*1.5):

- €1500 + 24.021 * €30 = €722.130,-

As can be seen, around 19.6 million euro can be saved on a year basis by using the biosensor instead of the CT scans, more tests with a more reliable diagnosis can be performed for less money.

Besides this, the total gain for the healthcare system is expected to be much larger, since the total costs for a patient with TBI that is part of the working population, direct healthcare costs were more than 3 times lower than the indirect costs (41). In addition, patients who receive the correct diagnosis can start treatment. This can result in less stress and anxiety about their health problems, which is very important since around 50% of adult patients with mild TBI who go to the hospital do not fully recover to pre-TBI levels of health in 6 months after their injury occurred (1). Moreover, it can result in less usage of medicine, and less long-term care with a lower disease burden (years of lost life (YLLs) and years lived with disability (YLDs)), while these patients can get to work again earlier and have less days of paid employment.

Appendix 5 Costumer Journey without and without NeuroTEST

Sophia is a 34-year old woman living in the Netherlands. After a sudden traffic accident on her way back from work, her life changes drastically. In the costumer journey maps, **Tables 4 and 5**, the process from accident to treatment is described without and with the presence of NeuroTEST.

Appendix 5A: current clinical practices

	Awareness	Consideration	Application	Treatment
Timeline	*~ 1 hour*	*~ 30 minutes*	*~ 2 hours*	*After 3.5 hours*
Persona: Sophia is 34-years old and lives a normal, healthy life **Scenario:** While cycling home from work, a car suddenly appears and does not have time to dodge. Sophia falls on her head **Goals & expectations:** Sophia needs an indication of the severity of her injury as soon as possible, to prevent further damage	Sophia's accident results in a fall directly onto her head. She lost consciousness, and experiences dizziness, dilated pupils and head ache when waking up. An ambulance is called, and brings her to the ER of the nearest hospital.	At the ER, a doctor analyzes Sophia's case by asking questions. Her symptoms are common for TBI, which is why the doctor decides to use the Glasgow Coma scale. From the questions in this scale, the doctor decides to prescribe a CT scan to detect TBI.	Sophia has to go to another room to get her CT scan made. Next, she has to wait for the doctor to interpret her results. When the doctor gets back, Sophia hears that no TBI could be seen, ruling out severe TBI. She gets the recommendation to keep lots of rest and to stay home for a while.	Sophia goes back home and takes as much rest as possible. However, after a certain amount of time has passed, she feels a little better and starts picking up things like work again. Her boss pressures her to start again as well, because no indication of serious damage was present. However, she continues to experience symptoms.
	Sophia is hurt and wants to feel better as soon as possible.	Sophia feels irritated and tired of the long process and questionnaire she has to undergo, and feels nervous while waiting for the results of her CT scan.	Sophia is disappointed and does not feel to be taken seriously. She starts doubting herself, and thinking she is just exaggerating.	Sophia feels pressured by all the expectations from her surroundings, and tired of working long days, while still having brain injury.

Table 4*: The costumer journey with the current clinical practices*

Appendix 5B: clinical practices with NeuroTEST

	Awareness	Consideration	Application	Treatment
Timeline	*~ 1 hour*	*~ 20 minutes*	*~ 10 minutes*	*After 1.5 hours*
Persona: Sophia is 34-years old and lives a normal, healthy life. **Scenario:** While cycling home from work, a car suddenly appears and does not have time to dodge. Sophia falls on her head. **Goals & expectations:** Sophia needs an indication of the severity of her injury as soon as possible, to prevent further damage.	Sophia's accident results in a fall directly onto her head. She lost consciousness, and experiences dizziness, dilated pupils and a head ache when waking up. An ambulance is called, and brings her to the ER of the nearest hospital.	At the ER, a doctor analyzes Sophia's case by asking questions. Her symptoms are common for TBI, but are not concrete enough to make a diagnosis. Therefore, the doctor decides to use the NeuroTEST biosensor.	The doctor takes a blood sample from Sophia and applies this to a cartridge of the **NeuroTEST**. **NeuroTEST** determines the severity of the TBI by taking into account the concentration of GFAP, the time after the accident and some personal parameters of the patient. A mild form of TBI is diagnosed.	The doctor can made a treatment plan at once, and Sophia can start her revalidation immediately. She has an accurate diagnosis thanks to the **NeuroTEST** and therefore does not try to go to work or do her chores, but instead takes the required rest and has a quicker and better recovery. Her boss allows her to stay home for a while, due to the clear diagnosis of the hospital.
	Sophia is hurt and wants to feel better as soon as possible.	Sophia feels tired and wants to know her diagnosis directly, without going to another doctor first.	Sophia is glad that she received an accurate and precise result in 10 minutes of time due to **NeuroTEST**.	Within 1,5 hours after the injury, Sophia can leave the ER. She feels relieved and thankful that the **NeuroTEST** provided a clear diagnosis this fast.

Table 5*: The costumer journey with the usage of NeuroTEST to diagnose TBI*

Appendix 6 SWOT analysis

It is important to assess the team's current position, since this can help by providing a clear overview of the team's current Strengths, Weaknesses, Opportunities, and Threats (SWOT). With this overview, the strategy needed to enter the market effectively can be improved. Moreover, the strengths the team possesses can be used in a more effective way, whilst also being aware of the weaknesses and the threats we may face. Then a plan can be made to improve our team, and by reducing the potential pitfalls. Consequently, the probability of achieving a successful market entry is increased.

Strengths

- The team consists of diverse members with different interests (chemistry, electronics, optics, programming, and business) which benefits the development of the sensor.
- T.E.S.T. 2023 is supported by a variety of companies, that open doors which could not be opened without them. These companies support us in goods, such as filters, but also with knowledge and advice. Moreover, these companies have an enormous network which will be beneficial for the future of the team and the start-up. All are truly enthusiastic about the development of the sensor.
- In addition, the team is supported by research groups at TU/e. These groups have a much knowledge about biosensors, and plasmon-enhanced fluorescence, and will be able to aid in the initial R&D when needed. The university is equipped to support start-ups by applying for patents and incorporate start-ups in the division TU/e participations including financial support.
- The team is very motivated, and eager to work to improve health care and people's quality of life. This motivation contributes to a healthy, and energetic working environment which is beneficial for the development for the sensor, as this is a major asset.
- T.E.S.T. made it to the final of the TU/e contest 2023, and won the ASML makers Award for the team's innovative and technological challenging idea and solution, which gave the team an enormous opportunity to present their biosensor to new investors and companies. The participation in this contest was of help for the business model of the sensor.
- NeuroTEST has great potential to be designed as a multiplex biosensor to measure multiple biomarkers at the same time, increasing the accuracy even more.

Weaknesses

- The students from T.E.S.T. are only from two different study programs (electronics and biomedical engineering). A more diverse team with for example students from chemical engineering, and computer science could be a major help to the team.
- The sensor does not measure in whole blood yet. Therefore, the blood still needs to be separated in order to measure in blood serum. This is an additional step in comparison with measuring in whole blood.
- For the assay, still a couple of handling steps are needed, which decrease the user-friendliness as a POC device. These steps need to be reduced to increase its usefulness.
- T.E.S.T. is relatively small and has limited resources (as compared to competing companies or startups).
- Point-of-care devices are still relatively new, so the implementation of such a biosensor will require some additional effort.
- There are already some patents on some of the components and approaches of the biosensor. This renders collaboration with partners and obtaining licenses more challenging.

Opportunities

- The market for TBI is increasing, since the population size is increasing as well.
- The awareness for TBI and its consequences and impact on our society are rising, which will be beneficial for the selling of the devices.
- The biosensor could be improved by making the materials sustainable and by doing research to make the production process more sustainable. Also research into the recyclability of the different components of the biosensor and cartridge could be performed to make the biosensor more sustainable.
- By creating this point-of-care biosensor, T.E.S.T. is contributing to the Sustainable Development Goal Health by ensuring healthy lives and promoting well-being at all ages.
- The biosensor can become a platform for new biosensor to measure every biomarker possible needed to diagnose other diseases by using other sandwich assay pairs.
- Other locations to implement the sensor could be explored, such as neurologists offices in other hospitals without an ER, or for example the military where many head injuries occur.

- Like the participation in the TU/e contest, T.E.S.T. will focus on the acquirement of a more extensive network by participating and presenting at markets and conferences, such as symposia, the Dutch design week, or the Health Demo Day.

Threats

- Other methods to diagnose TBI can form a hazard to our biosensor company, such as eye tracking or such as Banyan Biomarkers
- There is a second research group at the TU/e working on biosensors , the Merkx group, which can pose a threat when it comes to resources such as funding, exposure and experts. They can also pose as a competitor on the market of point-of-care rapid testing.
- The distribution of the biosensor to markets outside the Netherlands might pose a risk, since there might be different operating procedures of POCT's or other rules that make the distribution more difficult.
- Doctors can be quite conservative towards new treatment methods, since they know that their current method often function. Therefore, new methods need to be a 100% reliable and accurate. This could lead to them preferring current detection methods, like CT scans, making it hard to implement the biosensor in the clinic.
- The team is now reliant on the materials and facilities of the TU/e, which can lead to high pricing and material shortage. In the future, original manufacturers should be found to reduce the costs and increase the availability of the material.

Appendix 7 MOSCOW analysis

Must have	Should have	Could have	Will not have
• The ability to measure the GFAP concentration between 0.01 – 10.0 ng/ml • The ability to provide a reliable and accurate diagnosis of the severity of TBI • The ability to measure in blood plasma, which is easy to access when a patient needs to be rapidly tested • A maximum size of 80cm x 80cm x 50cm. • A casing preventing the laser light and electronics from being exposed to the user (as a laser of 250 mW is present) • The ability to perform at a maximum dilution ratio of 1:10 • A maximum sample volume of 20 µL per measurement	• As few handling steps as possible to have a high user-friendliness • Rounded outside corners to have a safe appearance • Hand-holds for easy transportation • A cost-effective design • A user-friendly interface • The ability to provide a diagnosis within 10 minutes • Reagents and consumables that have durable resistance during storage and use	• Sustainable materials • A future biosensor platform for other diseases with their own specific biomarkers • Materials that can be reused for newer versions of the sensor after an old version stopped functioning • The ability to measure multiple biomarkers (multiplex sensor) • A cartridge with a sugar coating for long-term storage • The inclusion of the time after the accident and some personal parameters in the result	• A zero CO_2 emission production process • Compatibility with non-specialized users • Haptic buttons • Bluetooth to connect with your phones or other devices • A wireless device design • A connection with an app

Appendix 8 Timeline NeuroTEST

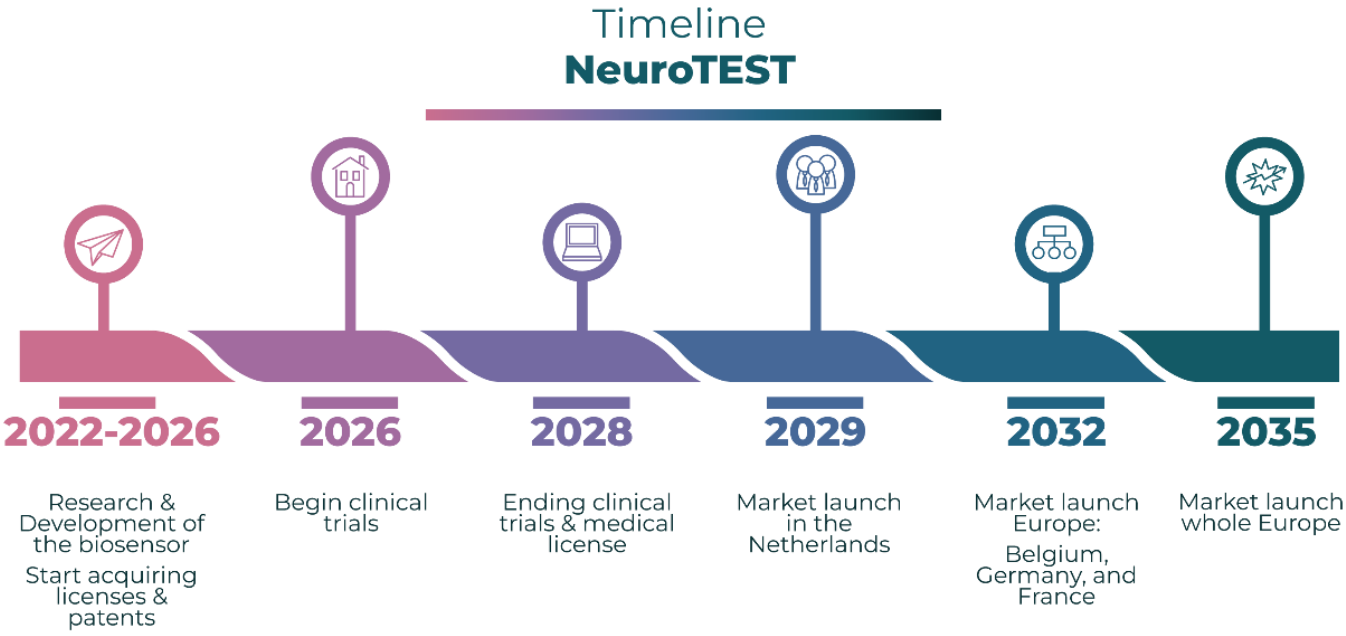


Figure 9: *Time of the NeuroTEST biosensor of T.E.S.T. from 2022 to 2035*

Appendix 9 Future devices of T.E.S.T.

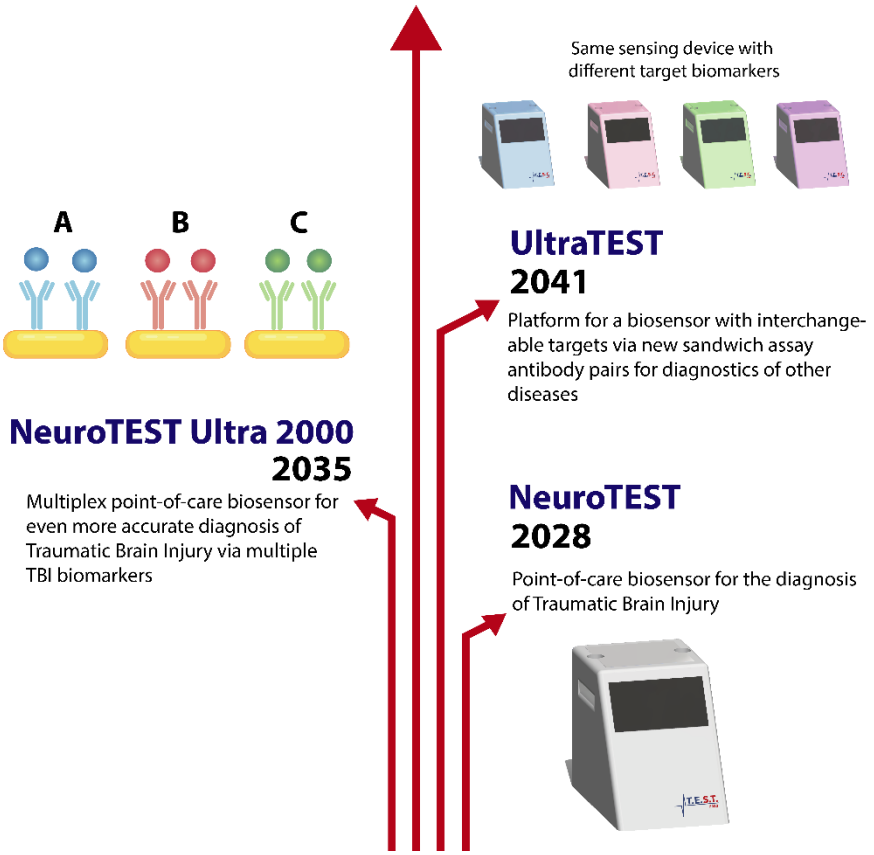


Figure 10: *Future devices of T.E.S.T.*

Appendix 10 Cost calculation cartridge

Table 6: *Cost overview of the total costs of cartridge*

Product	Price/ unit	Needed per cartridge	Price per cartridge
Nanorods NR-40-650 NanoSeedz	€218 / 10 mL of OD10	50 µL of OD1 (5 µL of OD10)	€0.109
Anti-GFAP 15cc capture antibody HyTest	€50 / mg	1 µL of 1 mg/mL	€0.05
Anti-GFAP 81cc detection antibody HyTest	€50 / mg	25 µL of 0.00015 mg/ml	€0.0001875
Atto 655 NHS ester	€385 / mg	10 µL (=1/100 of total) 2 mg/ml needed for 1 mg of antibody	€0.0000375
Traut's reagent	€96.60 / 100 mg	$9*10^{-6}$ g needed for 1 mg of antibody	€0.0000000088694
BSA	€70.10 / 10 g €2320 / 1 kg	0.05 µg	€0.000116
Microscope glass slides	€51.40 per 1000	1 per cartridge	€0.0514
Imaging spacers	$176 / 100 sheets of 8 stickers	1 per cartridge	$0.22 = €0.20
MPTMS	€43.60 / 25 g €478 / 500 g 1.057 g/mL at 25 °C (lit.)	250 µL of 10% v/v (=25 µL MPTMS =0.026 g) per batch of ±8 slides	€0.003107
Total costs			**€0.413848**

Appendix 11 Cost calculation prototype

Table 7: *Overview of the total costs of prototype*

Product	Price
Objective	€ 210
Camera	€ 670
Dichroic mirror	€ 220.22
Long pass filter	€ 398.09
Notch filter	€ 603.79
Laser	€ 19.80
Display	€ 80.00
Voltage circuit	€ 4.00
Raspberri pi	€ 60.00
3D printing costs	€ 50.00
Linear actuator	€ 144.21
Passive thermal mount	€ 431,68
Cables	€ 30
Total	**€ 2921.79**

Appendix 12 Cost estimation

It is expected that the final biosensor will cost 20% of the total costs of the prototype, which will result in a price of around €470,-. For the labor costs, it is expected that they will be around 10% of the raw production materials. Moreover, some additional costs for ICT, marketing, and distribution will be included in the sensor, which are expected to be 6% costs per device.

Table 8 *: Overview of production costs of prototype*

Costs	Price
Device costs	€470,-
Labor costs	€50,-
Marketing and distribution costs	€30,-
Total costs per device	**€550,-**

Appendix 13 Number of expected tests and cartridges sold

Yearly, 47.100 visit the ER in the Netherlands (14). Per patient, one test will be needed. There are 83 ERs in whole country (44). It is expected that at first not all ERs will buy the sensor, but it is expected that because of the marketing and networking before the launch, around 40 devices will be sold in the first year after launch. The years after more devices will be sold, since at first only ERs will be targeted but afterwards also hospitals without ERs will be focused on, which are 535 hospitals (618 - 83) (45). Especially after market launch in some countries of the EU, there will be a large increase in sold devices; therefor around 200 sensor will be expected to be sold in 2032.

Based on the amount of patients that visit the ER in the Netherlands (47.100), the amount of ER's, the average amount of patients per ER (47.100 / 83 = 567 patients) and the amount of devices sold in a year, the amount of sold cartridges were calculated. For this amount, it was taken into account that some patients who have TBI will unfortunately not be tested, while on the other side people who do not have TBI will receive a test when the doctor did not predict the potential cause of illness correctly.

Table 9*: Predicted amount of devices and cartridges sold in the period 2022-2032*

	2022-2026	2026-2028	2029	2030	2031	2032
# devices	0	0	35	55	95	200
# cartridges	0	0	20000	31000	53000	110000

Appendix 14 Cost and revenue overview

Year	2022-2026	2026-2028	2029	2030	2031	2032
Phase	R&D	Clinical trials	Market launch NL			Market launch EU
Fixed costs	(costs per year)					
Initial investment (R&D costs)	-€ 300.000,00	-€ 100.000,00	-€ 100.000,00	-€ 50.000,00	-€ 50.000,00	-€ 50.000,00
Medical license + clinical trials [a]	€ 0,00	-€ 125.000,00	-€ 2.000,00	-€ 2.000,00	-€ 2.000,00	-€ 2.000,00
Intellectual Propery costs (patent, tm, etc.) [b]	-€ 30.000,00	-€ 30.000,00	-€ 40.000,00	-€ 40.000,00	-€ 55.000,00	-€ 75.000,00
Rent [c]	-€ 54.000,00	-€ 54.000,00	-€ 54.000,00	-€ 54.000,00	-€ 54.000,00	-€ 99.000,00
Quality assurance [d]	€ 0,00	€ 0,00	-€ 50.000,00	-€ 100.000,00	-€ 100.000,00	-€ 200.000,00
Total	-€ 384.000,00	-€ 309.000,00	-€ 246.000,00	-€ 246.000,00	-€ 261.000,00	-€ 426.000,00
Variable costs						
Costs per device	-€ 550,00	-€ 550,00	-€ 550,00	-€ 550,00	-€ 550,00	-€ 550,00
# devices	0	0	40	70	120	200
Total costs devices	€ 0,00	€ 0,00	-€ 22.000,00	-€ 38.500,00	-€ 66.000,00	-€ 110.000,00
Costs per cartridge	-€ 0,20	-€ 0,20	-€ 0,20	-€ 0,20	-€ 0,20	-€ 0,20
# cartridges	0	0	15000	26250	50000	90000
Total costs cartridges	€ 0,00	€ 0,00	-€ 3.000,00	-€ 5.250,00	-€ 10.000,00	-€ 18.000,00
Salary per employee [e]	-€ 50.000,00	-€ 50.000,00	-€ 70.000,00	-€ 70.000,00	-€ 70.000,00	-€ 70.000,00
Salaries	-€ 550.000,00	-€ 550.000,00	-€ 1.400.000,00	-€ 1.400.000,00	-€ 1.400.000,00	-€ 1.400.000,00
Marketing	-€ 5.000,00	-€ 75.000,00	-€ 75.000,00	-€ 100.000,00	-€ 150.000,00	-€ 200.000,00
Total	-€ 555.000,00	-€ 625.000,00	-€ 1.500.000,00	-€ 1.543.750,00	-€ 1.626.000,00	-€ 1.728.000,00
Total costs	-€ 939.000,00	-€ 934.000,00	-€ 1.746.000,00	-€ 1.789.750,00	-€ 1.887.000,00	-€ 2.154.000,00
Revenue						
Per device	€ 0,00	€ 0,00	€ 1.500,00	€ 1.500,00	€ 1.500,00	€ 1.500,00
# devices	0	0	35	55	95	200
Total revenue from devices	€ 0,00	€ 0,00	€ 52.500,00	€ 82.500,00	€ 142.500,00	€ 300.000,00
Per cartridge	€ 0,00	€ 0,00	€ 30,00	€ 30,00	€ 30,00	€ 30,00
# cartridges	0	0	20000	31000	53000	110000
Total revenue from cartridges	€ 0,00	€ 0,00	€ 600.000,00	€ 930.000,00	€ 1.590.000,00	€ 3.300.000,00
Maintenance	€ 0,00	€ 0,00	€ 25.000,00	€ 35.000,00	€ 50.000,00	€ 80.000,00
Funding & grants	€ 200.000,00	€ 150.000,00	€ 0,00	€ 0,00	€ 0,00	€ 0,00
Investments	€ 250.000,00	€ 275.000,00	€ 500.000,00	€ 450.000,00	€ 250.000,00	€ 0,00
Total revenue	€ 450.000,00	€ 425.000,00	€ 1.177.500,00	€ 1.497.500,00	€ 2.032.500,00	€ 3.680.000,00
Net income	**-€ 489.000,00**	**-€ 509.000,00**	**-€ 568.500,00**	**-€ 292.250,00**	**€ 145.500,00**	**€ 1.526.000,00**

[a] clinical trials & medical license average
[b] Patent = European Patent = Filing Fees + Search and Examination Fees + Publication Fees + Maintenance fees
[c] Rent = office + lab facilities = 24.000 + 30.000 (€240,- per m2 --->100 m2)
[d] Quality assurance needed to maintain the biosensor's quality
[e] Salary based on (Biomedical Engineer salary in Netherlands in 2023 | PaSyScale, 2023)

Table 10: *Estimated revenue and cost projection and net income for 2022-2032*

Appendix 15 Estimated revenue, cost projection and net income

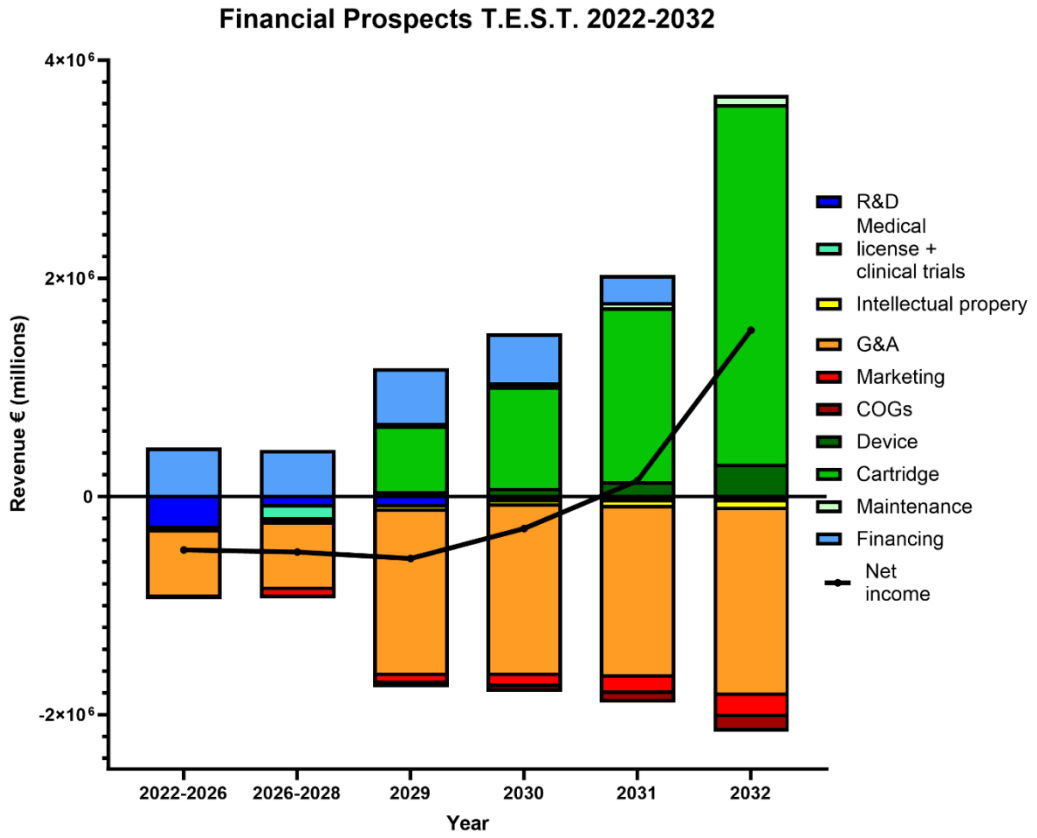


Figure 11 : *Estimated revenue, cost projection and net income for T.E.S.T. in the period of 2022 until 2032*

Appendix 16 Survey sent to 4 people with concussion

This small survey was sent to acquaintances of the team, who have experienced a head injury to get more insight into their symptoms, behavior, and whether they thought a sensor could have additional.

1. Hoe ben je gevallen?
2. Ben je bewusteloos geweest?
3. Waar heb je last van gehad in de dagen na je val?
4. Ben je naar de huisarts geweest? Waarom wel of niet?
    a. Zo ja wat was het advies van de huisarts?
    b. Zo ja, hoe is de diagnose gesteld?
    c. Zo ja, hoe lang duurde de hele diagnose en/of bezoek?
5. Hoe lang heb je last gehad van je val?
6. Heb je rust gehouden na je val?
7. Heb je het gevoel dat je inmiddels helemaal hersteld bent van het letsel?
8. Heb je veranderingen gemerkt in alertheid, spreken, coördinatie of andere tekenen van letsel?
9. Had je een bloedsample willen afstaan om met een biosensor de heftigheid van het letsel te meten?
10. Zou jij toegevoegde waarde zien in een sensor die kan meten hoe erg het letsel is?
11. Heb je nog andere dingen die je zou willen vertellen over je val of over de symptomen die je daarna had?

Appendix 17 Social impact

By measuring concentrations of GFAP from 0,01 until 10 ng/mL, the lightest forms of TBI as well as the most severe cases can be detected and distinguished. This contributes both to the cost reduction and increase of accessibility; by distinguishing light, moderate and severe cases, CT scans can be preserved for only the more severe ones of which a CT scan is likely to show the damage. The less severe cases of TBI can be filtered out before the scan has been made. On the one hand this reduces the amount of CT scans to only the most urgent and useful ones, which reduces costs, and on the other hand this provides an indication of the severity for all cases of TBI that cannot be shown in current scans. Due to this, more patients can receive an accurate diagnosis, allowing doctors to provide them with a specific treatment plan resulting in the best possible recovery.
Furthermore, with the NeuroTEST being present in the ER, it can save many patients the step of being referred to the hospital for an actual scan, which is beneficial for both accessibility of the diagnosis for a patient and for reduction of the costs for the hospital management. Moreover, this can prevent the issue of ER overcrowding by helping patients faster. Due to the high occurrence rates of TBI, we believe that NeuroTEST can be highly significant for the development of healthcare, and can have an important societal impact on many patients in the future.