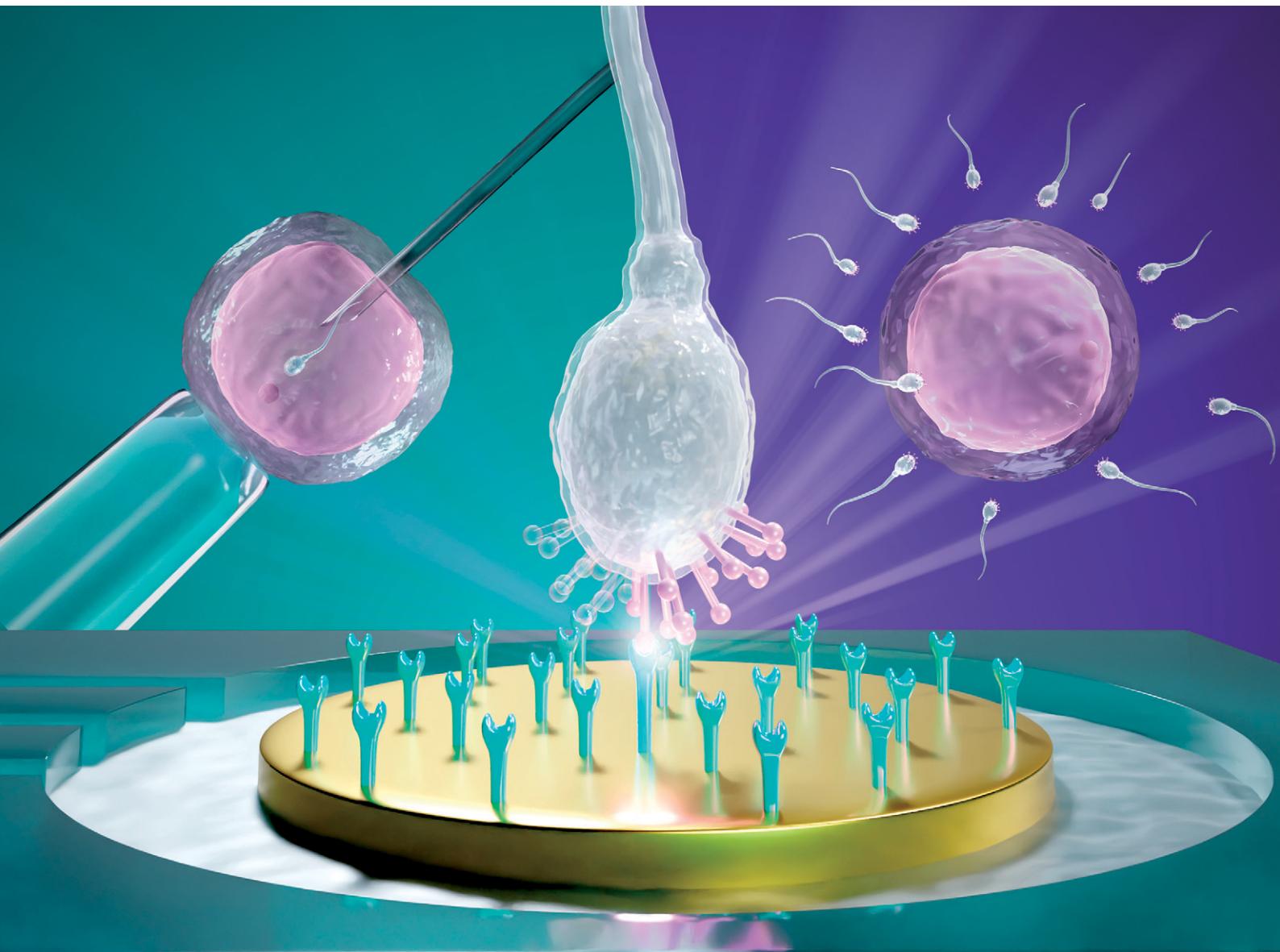


Sensors & Diagnostics

rsc.li/sensors



ISSN 2635-0998



Cite this: *Sens. Diagn.*, 2025, **4**, 579

JUNO-Checked – a live cell electrochemical biosensor for sperm function diagnostics†

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Conventional diagnosis for male-factor infertility primarily comprises standard microscopic semen analysis, including sperm count or concentration, motility, and morphology of sperm. Unfortunately, this standard analysis offers limited predictive value for pregnancy or the success of assisted reproduction treatments. There is an urgent need for diagnostic tools that effectively probe sperm function. This study discloses a novel diagnostic tool, JUNO-Checked, comprising an electrochemical biosensor for sperm function inspired by the molecular mechanisms of fertilization, utilizing immobilized JUNO protein of oocyte origin. We show that the JUNO-Checked biosensor can quantify sperm binding to the JUNO functionalized biosensor, represented here as the JUNOScore. This score provides a unique insight into sperm function that is inaccessible through standard microscopic semen analysis. Our results underscore the novelty and potential utility of the JUNO-Checked biosensor in clinical settings for diagnostics and personalized assisted reproduction treatments.

Received 22nd January 2025
 Accepted 13th March 2025

DOI: 10.1039/d5sd00009b

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Introduction

The limitations of standard semen analysis have long-constrained diagnosis and laboratory assessment of male-factor infertility. Since 1980, the World Health Organization (WHO) has provided guidelines for standard semen analysis that take sperm count or concentration, motility, and morphology into account and have remained unchanged for several decades.¹ While informative, standard semen analysis is known to have limited value for the prognosis of fertility outcomes for natural pregnancy or success of assisted reproduction treatments (ART).^{2,3} Given the alarming rate at which sperm count and concentration have fallen globally in the last five decades, and even more so in recent years,^{4,5} there is a pressing need for the incorporation of sperm function analysis in laboratory diagnosis of male-factor infertility, as well as personalization of ART.^{6,7}

Advanced tests for sperm function analysis have long existed within academic environments, primarily focusing on sperm DNA integrity, acrosome status, and the ability of sperm to bind to human or animal oocytes.^{8–10} Recently, an

electrochemical diagnostic device that probes the redox potential of seminal plasma to quantify the level of oxidative stress has also been described and commercialized as the MiOXSYS system.¹¹ However, clinical implementation of these diagnostic methods is contentious due to conflicting clinical evidence and lack of standardization.^{8,12–14}

The last decade has seen increasing evidence and expert consensus for the implementation of sperm function tests, especially the analysis of sperm DNA fragmentation in clinical practice for both ART and pregnancy loss.^{15–17} Despite these advances, sperm function tests that can effectively assess the ability of sperm to bind to and fertilize the oocyte have mainly existed in academic literature,^{6,18} including the hemizona assay, which has shown promising results for fertility outcomes in meta-analyses.⁸ Since sperm function tests assess the fertilization competence of sperm, they have the potential to help create personalized ART regimens by assisting with a reasonable selection of the appropriate treatments among intra uterine insemination (IUI), standard IVF (stIVF) and/or intra cytoplasmic sperm injection (ICSI).¹⁸

To develop a sperm function diagnostic tool, we resorted to inspiration from the molecular mechanisms underlying human fertilization. A functional sperm–oocyte interaction requires sperm to undergo an acrosome reaction, wherein the acrosome membrane, an anterior organelle in the sperm-head, fuses with the sperm plasma membrane. Subsequently, during sperm–egg interaction, the sperm IZUMO protein

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d5sd00009b>



superfamily, that relocates to the sperm head outer membrane prior to fertilization,^{19,20} is critically involved in sperm oocyte fusion.²¹ With the discovery of oocyte membrane protein JUNO as the binding partner of sperm IZUMO1, it was shown that JUNO IZUMO1 pairing is the critical step for fertilization in rodents²² and humans.²³

Further, independent research groups have reported the involvement of the IZUMO1 dysregulation and phosphorylation in malefactor infertility^{24,25} thereby underscoring the JUNO sperm-binding as the ideal sperm-function test to assess the fertilization competence of sperms. It is important to note that sperm binding to JUNO has been shown to be a good predictor for bovine fertility, a model animal to study fertility.²⁶

In the current work, we disclose a JUNO-containing electrochemical biosensor for sperm function assessment, coined the JUNO-*Checked* biosensor (Fig. 1).²⁷ The JUNO-*Checked* biosensor can detect IZUMO1 in solution, as well as through a semi-automated analysis, to evaluate sperm–JUNO binding as a semi-quantitative JUNOScore. While lab-on-a-chip assays for analysis of seminal plasma¹¹ or semen concentration and motility²⁸ have been previously described, to the best of our knowledge, JUNO-*Checked* is the first live-cell electrochemical biosensor that analyses the binding of motile mammalian cells to a gatekeeper membrane protein. Clinical semen samples were also tested with the JUNO-*Checked* biosensor, which aims to provide complementary information to standard semen analysis for personalized ART treatments.

Materials and methods

Materials

Dithio-bis(succinimidyl propionate) (DSP), tris(2-carboxyethyl) phosphine (TCEP), dimethyl sulfoxide (DMSO), phosphate

buffered saline, 2-(*N*-morpholino)ethanesulfonic acid (MES buffer), *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES buffer), sodium chloride (NaCl), casein hydrolysate, α,α -trehalose and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Merck Life Science UK Limited or Merck Life Science AB, and were of at least MQ200 quality grade. Recombinant human proteins from mammalian hosts, including JUNO, IZUMO1, and biotinylated-IZUMO1 (biotin-IZUMO1), and thin-film gold three-electrode sensors were purchased from FlexMedical Solutions Ltd (UK). Invitrogen's horseradish peroxidase-conjugated streptavidin (strep-HRP), Fujifilm Irvine Scientific's continuous single culture complete with gentamycin and HSA (CSCM-C) were purchased from Thermo Fisher Scientific (UK), Novovitae (Denmark), respectively.

Development of JUNO-*Checked* biosensor

Bare gold sensors were functionalized by creating a self-assembled monolayer with DSP and TCEP. Briefly, stock solutions of DSP in DMSO and TCEP in MES were diluted in a solution containing 10% DMSO and 90% MES buffer. The final solution containing 1 mM DSP and 2 mM TCEP was added to the electrodes to fully cover the sensor surface, followed by 1 h incubation, rinsing with MES buffer and tapping dry. 5 μ g mL⁻¹ JUNO, in a solution containing 10 mM HEPES and 140 mM NaCl, was incubated for 1 h, followed by rinsing with HEPES buffer. Finally, the biosensors were blocked using 1 mg mL⁻¹ hydrolyzed casein dissolved in PBS for 1 h, then rinsing with PBS. All incubations were at room temperature unless specified. Lastly, the drying buffer (50 mM Tris, 100 mM glycine, 500 mM NaCl, pH 7.5, 15% trehalose) was added to

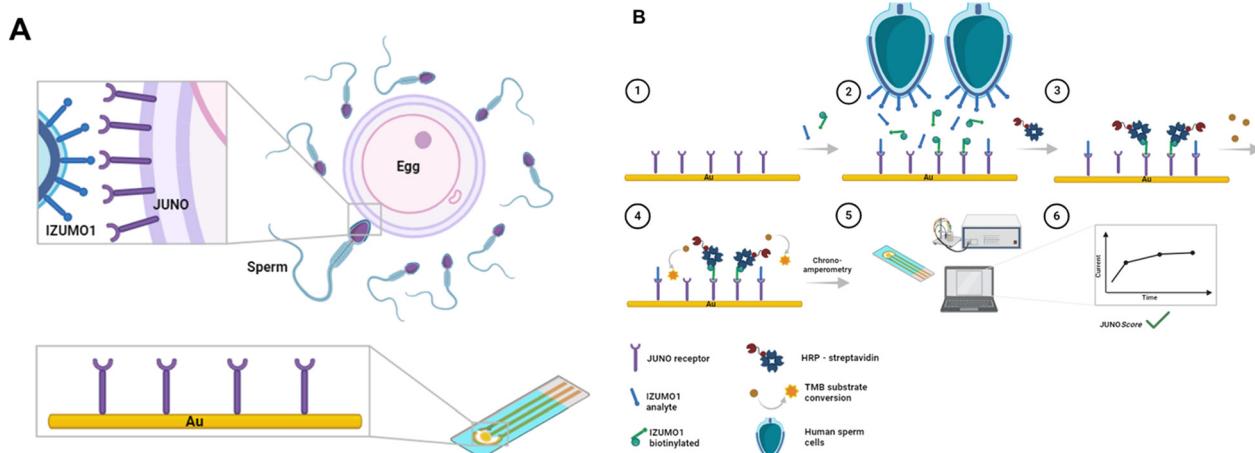


Fig. 1 Design of the JUNO-*Checked* biosensor and assay: A. schematic representation of the sperm IZUMO1 and oocyte JUNO interaction during the fertilization process; B. JUNO-*Checked* biosensor assay design wherein¹ the biosensor comprises of a gold transducer and surface-immobilized recombinant human JUNO as the biorecognition element.² The JUNO-*Checked* biosensor is incubated with the competing agent – biotinylated-IZUMO1 (biotin-IZUMO1), and the analyte of interest – IZUMO1 or human sperms. After incubation,³ streptavidin-horseradish peroxidase (strep-HRP) conjugate is allowed to detect the remaining competing agent, followed by⁴ addition of and reaction with the HRP substrate, 3,3',5,5'-tetramethylbenzidine (TMB).⁵ The electrochemical readout is enabled via chronoamperometry with a potentiostat setup, and⁶ the binding is visualized as the relative displacement of the competing agent in terms of current values or JUNOScore.



the electrode surface and dried at 40 °C for two hours. This drying buffer was used to maintain both the structure and function of the sensor and to allow for the stability of the active proteins for an extended period.

A direct enzymatic assay was performed with biotin-IZUMO1, strep-HRP, and TMB to assess the functionality of the JUNO-*Checked* biosensor. To this end, biosensors were washed with 2 mL PBS and gently tapped dry. 70 µL of the biotin-IZUMO1 were added to the electrode surface and incubated for 1 h. Various concentrations of biotin-IZUMO were tested (250, 500, and 1000 ng mL⁻¹). Upon rinsing with 1 mL PBS, 70 µL strep-HRP was added and incubated for one hour. After the incubation, the electrodes were washed with PBS, and 70 µL TMB was added and incubated in the dark for 20 min.

The biosensors were connected to the MultiPalmsSens potentiostat (PalmSens, The Netherlands) to perform a chronoamperometric analysis for 60 s at an applied potential of -0.2 V.

Clinical sample collection, analysis and processing

This study exclusively used leftover sperm samples from donors who had already provided informed consent for their samples to be used in research. Malmö Reproductive Medicine Center managed the consent process following national regulations and ethical guidelines.

The samples were fully anonymized at the point of collection, with no direct or indirect identifiers and no possibility of re-identification. This distinction is critical, as it determines the ethical and regulatory requirements for human biological material research.

According to the Swedish Ethical Review Authority (Eтикпрöвнingsmyndigheten), studies using fully anonymized human samples without connection to the donor exist and do not require formal ethical approval. Furthermore, all samples were handled strictly in line with national and institutional guidelines, used solely for the intended analysis, and immediately discarded thereafter under standard protocols for biological material.

Leftover, anonymized semen samples were received after standard diagnostic analysis from healthy volunteers among patients visiting the Andrology Lab, Reproduktionsmedicinskt centrum, Skåne University Hospital, Malmö. Only volunteers aged 23–45 years, in good general health, and free from any known reproductive or sexual health disorders, were included in the study. Further, semen samples that qualified the following parameters were included: successful liquefaction between 15–60 min, under rocking at 37 °C; total semen ejaculate volume between 1–8 mL; sperm concentration $\geq 16 \times 10^6$ mL⁻¹; progressive motility $\geq 30\%$; and leftover semen sample volume ≥ 500 µL. The liquefied semen samples were received and used immediately.

Sperm concentration and motility were assessed upon receipt of a liquefied semen sample and after simple washing. Briefly, 3 µL of semen or sperm sample was added

to a Counting Chamber SCA (Microptic S.L., Spain) and was analyzed using a Nikon Ci-L microscope at 10 \times magnification under positive phase contrast, in conjunction with SCA Morphology module (Microptic S.L., Spain).

Liquefied leftover semen samples were processed with simple washing to remove the seminal plasma as described elsewhere.²⁹ Briefly, 500 µL liquefied leftover semen samples were washed twice by diluting in 500 µL CSCM-C at 37 °C, followed by centrifugation at 500g for 10 min. The pellet was finally resuspended in 500 µL CSCM-C. The washed sample was then assessed for concentration and motility as described earlier, followed by adjusting the concentration of the sperm samples to 5, 10, 15, and 20×10^6 mL⁻¹ in CSCM-C.

IZUMO1 detection and sperm binding assay on JUNO-*Checked* sensor

For the competitive chronoamperometric assay on the JUNO-*Checked* biosensors, IZUMO1 analyte at 50, 125, 250, 500, or 1000 ng mL⁻¹ concentrations in PBS, or washed sperm samples at the desired concentration were used as the analyte solutions. As described earlier, the biosensors were washed to remove the drying buffer. The analyte solutions were dispensed to JUNO-*Checked* biosensors and incubated for 30 min at 37 °C, followed by washing with 2 mL of PBS and gently tapping dry. A fixed concentration of 125 ng mL⁻¹ biotin-IZUMO1 was added to the sensors as a competing agent and incubated for 60 min. The sensors were flushed twice with 1 mL PBS and gently tapped dry. 70 µL strep-HRP was dispensed on the electrode surface, followed by 60 min incubation and washing twice with 1 mL PBS. Finally, 70 µL TMB was added to the electrodes and incubated in the dark for 20 min. All incubations were at room temperature unless specified. Chronoamperometric analysis at an applied potential of -0.2 V was performed with the MultiPalmsSens potentiostat (PalmSens, The Netherlands).

Results and discussion

Development of the JUNO-*Checked* sensor

The JUNO-*Checked* biosensor is intended to assess the fertilization competence of sperms in a clinical setting, *i.e.*, the test should measure the binding of JUNO-containing biosensors to sperm cells that are alive. However, developing an assay for living cells poses technical challenges, as the assay needs to be both specific and gentle. To accomplish this, we combined chronoamperometry and a competitive enzymatic-protein-linked assay. The performance of the assay design was initially tested in a direct enzymatic chronoamperometric assay using the binding of biotin-IZUMO1 to the JUNO-*Checked* biosensor, where the chronoamperometric reporting was enabled through the enzymatic reaction between the cognate strep-HRP and the TMB substrate.

As HRP oxidizes TMB, a reduction potential was applied during the electrochemical analysis to reduce the oxidized TMB species at the working electrode. This allowed for the detection of the oxidized TMB, thereby reflecting the amount



of the cognate step-HRP bound to the biotin-IZUMO1 immobilized on the JUNO-*Checked* biosensor surface. As expected, the observed current in chronoamperometry steadily increased with time for each of the tested concentrations of biotin-IZUMO1, reaching a plateau of the measured current within 60 s (Fig. 2A). This behavior can be attributed to the diffusion of the oxidized TMB species to the electrode surface. Further, the magnitude of the current response value observed at $t = 60$ s increased exponentially ($R^2 = 0.99$) with the increasing concentration of biotin-IZUMO1 (Fig. 2B), indicating successful binding of IZUMO1 to JUNO immobilized on the sensors.

IZUMO1 detection using the electrochemical JUNO-*Checked* biosensor

The performance of the chronoamperometric competitive assay on the JUNO-*Checked* biosensor was tested at various concentrations of the IZUMO1 analyte, wherein biotin-IZUMO1 was used as a competing agent. As before, the chronoamperometric analysis allowed for the detection of the oxidized TMB, thereby reflecting the amount of the cognate step-HRP bound to the biotin-IZUMO1 tethered on the JUNO-*Checked* biosensor surface. Thus, similar to the previous direct enzymatic assay, the observed current in chronoamperometry steadily increased with time for each experiment, eventually reaching a plateau (Fig. 2C).

However, given the assay was designed for IZUMO1 analyte and biotin-IZUMO1 to compete for the available JUNO at the biosensor surface, with increasing concentrations of IZUMO1 analyte at a fixed biotin-IZUMO1 concentration, the magnitude of the current response value observed at $t = 60$ s decreased exponentially with the increasing concentration of IZUMO1 analyte (Fig. 2D). This may be attributed to the binding of IZUMO1 analyte to the immobilized JUNO on the biosensor surface, leading to effective displacement of biotin-IZUMO1 (or prevention of its binding) and consequently strep-HRP, thereby leading to a decrease in HRP-TMB redox reporting. This dose-dependent effect followed an exponential trend with $R^2 = 0.91$ (Fig. 2D).

The binding of the IZUMO1 analyte was quantified in terms of the relative displacement of biotin-IZUMO1, measuring the relative decrease in current response value observed at $t = 60$ s, normalized to a null concentration of IZUMO1 (Fig. 2E). Relative displacement was found to be directly correlated with increasing concentration of IZUMO1 ($R^2 = 0.88$), thereby demonstrating the ability of the JUNO-*Checked* biosensor to detect IZUMO1 in solution effectively.

Detection of human sperm cells with the JUNO-*Checked* biosensor

After demonstrating a dose-dependent response of the JUNO-*Checked* biosensor against the purified human sperm

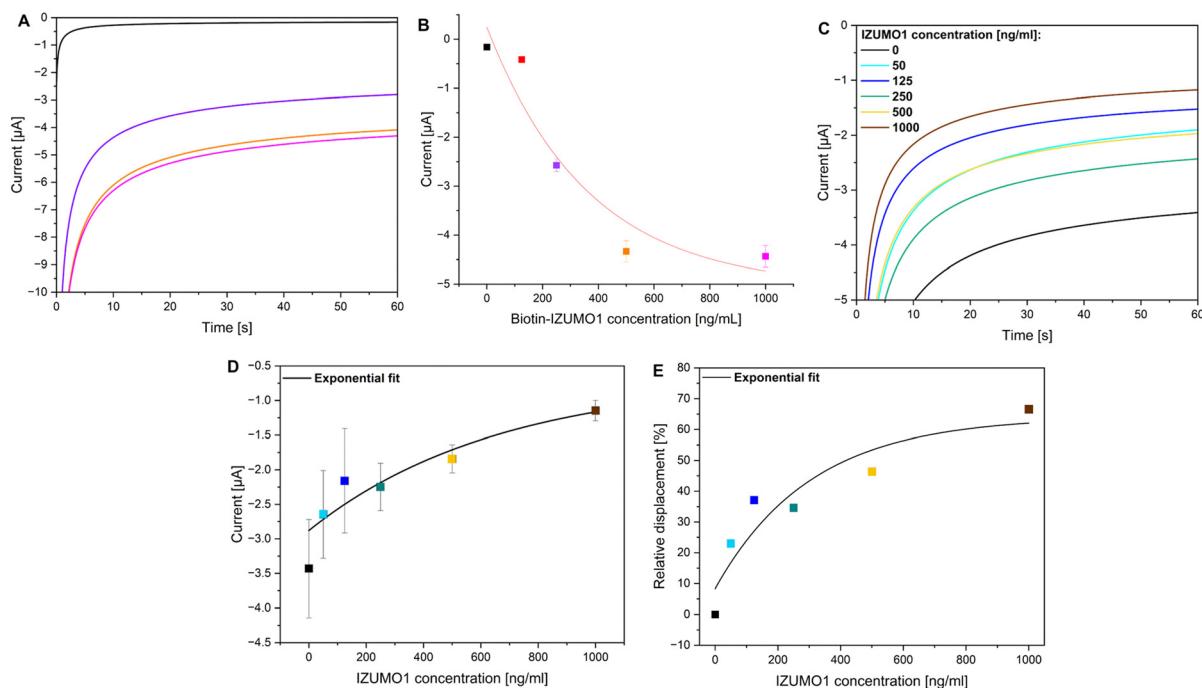


Fig. 2 Performance of JUNO-*Checked* biosensor with protein analyte: A. chronoamperometric response in the direct enzymatic assay at different biotin-IZUMO1 concentrations and redox reporting using strep-HRP and TMB in solution; B. decrease in current response values at $t = 60$ s for increasing biotin-IZUMO1 concentrations in the direct enzymatic assay; C. chronoamperometric response of the sensor at different IZUMO1 concentrations in the competitive assay, with biotin-IZUMO1 as the competing agent, and redox reporting using strep-HRP and TMB in solution; D. increase in current response values at $t = 60$ s for increasing concentrations of IZUMO1 in the competitive assay; E. visualization of sensor response to IZUMO1 binding as relative displacement of biotin-IZUMO1 at different IZUMO1 concentrations ($n = 7$ or 8).



IZUMO1 protein, the biosensor's performance was tested for sperm binding in clinical semen samples. For this purpose, sperm samples were adjusted to the following concentrations: 5, 10, and $20 \times 10^6 \text{ mL}^{-1}$ (for details, see Materials and methods) and assayed with the competitive assay protocol using the biotin-IZUMO1 as the competing agent and redox reporting through the HRP-TMB reaction.

Remarkably, the sensor showed similar behavior as with the IZUMO1 analyte protein. The current, measured through chronoamperometry, showed a steady increase as expected, with a plateau at approximately $t = 60 \text{ s}$ (Fig. 3A). Presumably indicating upon binding of the sperm cells to the JUNO-*Checked* biosensor, sperms were also observed to displace biotin-IZUMO1 and strep-HRP. Consequently, with higher concentrations of sperms, a lower magnitude of current was observed at an arbitrary time point (Fig. 3A). The electrochemical response of sperm-binding on the JUNO-*Checked* biosensor also showed an exponential trend for current values at $t = 60 \text{ s}$ ($R^2 = 0.99$, Fig. 3B). These values were represented as relative displacement of biotin-IZUMO1 by the sperms by normalizing the relative decrease in sensor current response value at $t = 60 \text{ s}$ for null concentrations (Fig. 3C).

While averaged relative displacements were found to be directly correlated with the sperm concentrations, each clinical semen sample was attributed with a numerical coefficient called JUNOScore (Fig. 3D), calculated as its respective relative displacement at the fixed sperm

concentration of $10 \times 10^6 \text{ mL}^{-1}$, and normalized on a scale of 0–10. Here, JUNOScores 0 and 10 correspond to a theoretically null and 100% relative displacement of biotin-IZUMO1 in the competitive assay, indicating poor and excellent sperm-JUNO binding, respectively. Interestingly, a significant spread of JUNOScores was found in different semen samples, shedding light on the heterogeneity of the binding capacity of sperms to the oocyte protein JUNO.

To investigate a relationship between standard semen analysis parameters and the JUNOScore, we have measured the concentration and motility of the sperm samples (details in Materials and methods). Surprisingly, sperm concentration (Fig. 3E) and progressive motility (Fig. 3F) did not correlate with JUNOScore, thereby elucidating the uniqueness of JUNOScore owing to the testing of JUNO-binding in the assay design. The lack of correlation between a sperm-JUNO binding dependent JUNOScore and the WHO-recommended standard semen parameters further underscores the novelty and potential utility of the JUNOScore in clinical settings as a complementary diagnostic measure to the standard semen analysis.

Conclusions

Since the publication of the first WHO manual for semen analysis in 1980, microscopic analysis of sperm concentration, motility, and morphology has been the foundation of diagnosis for male-factor infertility. While we

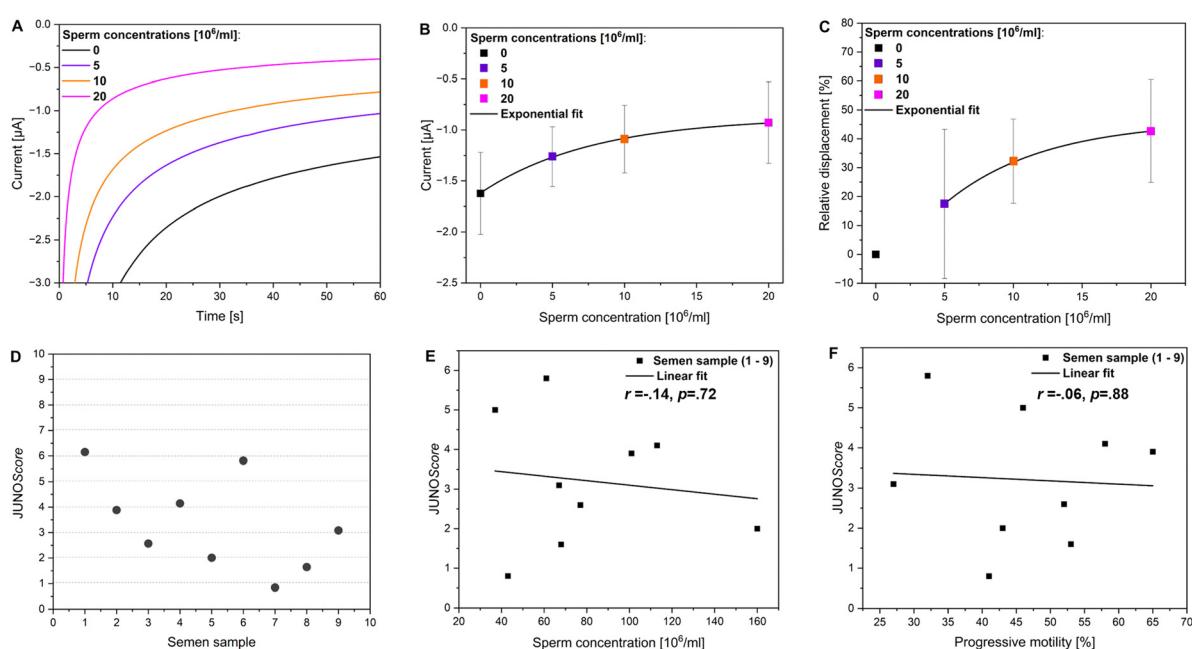


Fig. 3 Competitive assay of living sperm cells with the JUNO-*Checked* biosensor. A. Representative chronoamperogram of a JUNO-*Checked* biosensor with different sperm concentrations, with biotin-IZUMO1 as the competing agent and redox-reporting using strep-HRP and TMB in solution B. Current value at 60 s from the plots in (A) at various sperm cell concentrations. The solid line for exponential fitting; C. sperm binding represented the relative displacement of biotin-IZUMO1. The calculation is based on averaged current responses for 9 clinical sperm cell samples; D. sperm-binding shown as JUNOScore from clinical sperm samples ($n = 9$); and comparison of JUNOScores with the sperm concentration, E and progressive motility, F and includes the values for Pearson's correlation coefficient, r , and the probability value, p .



have learned that sperm count and concentration have been declining rapidly in the last five decades, microscopic semen analysis has shown clear limitations in yielding clinically helpful information for the prognosis of pregnancy or the success of ART. Thus, there is an urgent need for improving the diagnostics for male-factor infertility.

The current work has introduced a significant advancement in reproductive medicine by developing a JUNO-*Checked* biosensor to detect JUNO-IZUMO1 binding—a gatekeeper step in oocyte fertilization. Inspired by the specific interaction during sperm–oocyte fusion, we have demonstrated a biosensor that can detect different concentrations of IZUMO1 in solution by allowing *in vitro* JUNO-IZUMO1 binding. By use of clinical semen samples, we have further shown that the JUNO-*Checked* biosensor enables live-cell detection of motile sperms by on-chip capture and can be used for direct assessment of sperm's ability to engage in the fertilization process.

Our findings reveal that the JUNOScore, derived from electrochemical analysis of sperm-JUNO binding on the surface of the JUNO-*Checked* biosensor, presents unique information that does not correlate with conventional semen analysis of the ejaculate semen sample, including sperm concentration and motility. This lack of correlation underscores the potential of JUNOScore to serve as a novel diagnostic parameter, offering insights into male-factor infertility that were previously inaccessible through standard laboratory evaluations. Future studies shall address the effect of media and matrix on the performance of JUNO-*Checked* biosensor and JUNOScore, as well as their utility in predicting the success of fertilization in stIVF and other treatment outcomes.

In conclusion, integrating complementary biosensor technologies, like the JUNO-*Checked* biosensor, in the assessment of sperm-function offers a promising avenue in the field. This would pave the way for enhanced first-line diagnostic precision, shed light on idiopathic and unexplained male infertility, and facilitate personalized assisted reproduction treatments.

Data availability

The datasets supporting this article have been uploaded as part of the ESI.[†]

Author contributions

K. P.: conceptualization, methodology, funding acquisition and writing, I. L. S.: supervision and investigation, M. L.: resources, supervision, and investigation, P. P.: investigation, F. L.: investigation, T. D.: funding acquisition resources, C. P.: formal analysis, visualization, and writing, D. F.: resources and data curation, J. C. L.: visualization, data curation, and writing; J. S.: conceptualization, investigation, data curation, and writing.

Conflicts of interest

Kushagr Punyani is a founder and owns stock in Spermsons AB and Nested Bio. Maria Liljander, Panchami Pradeepkumar, Jaime Castillo-León, Frida Lunblad, Carolin Psotta, Jae Shin, and Tore Duvold have been employees or consultants at Spermsons AB and may own/have owned stocks or other financial instruments in Spermsons AB. Kush Punyani and Ingela Liljeqvist Soltic are board members of Spermsons AB.

Acknowledgements

This work received Vinnova funding under the 2023-00913, 2022-01404, 2021-02367, 2021-02133, and 2019-00234. The authors thank Professor Aleksander Giwercman (Lund University and Reproduktionsmedicinskt centrum, Sweden), Dr. Emir Henic (Reproduktionsmedicinskt centrum, Sweden), Prof. Trine B. Haugen (Oslo Metropolitan University, Norway), and Prof. Claus Yding Andersen (Rigshospitalet, Denmark) for their advice in planning the study.

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