# A biophotonic approach to measure pH in small volumes *in vitro*: quantifiable differences in metabolic flux around the cumulus-oocyte-complex (COC).

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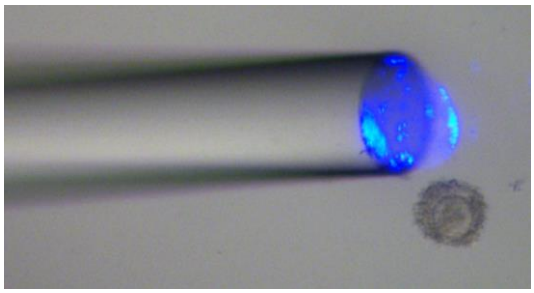
Unfertilised eggs (oocytes) release chemical biomarkers into the medium surrounding them. This provides an opportunity to monitor cell health and development during assisted reproductive processes if detected in a non-invasive manner. Here we report the measurement of pH using an optical fibre probe, **OFP1**, in 5 µL drops of culture medium containing single mouse cumulus-oocyte-complexes (COCs). This allowed for the detection of statistically significant differences in pH between COCs in culture medium with no additives and those incubated with either a chemical (cobalt chloride) or hormonal treatment (follicle stimulating hormone); both of which serve to induce the release of lactic acid into the medium immediately surrounding the COC. Importantly, **OFP1** was shown to be cell-safe with no inherent cell toxicity or light-induced phototoxicity indicated by negative DNA damage staining. Pre-measurement photobleaching of the probe reduced fluorescence signal variability, providing improved measurement precision (0.01 – 0.05 pH units) compared to previous studies. This optical technology presents a promising platform for the measurement of pH and the detection of other extracellular biomarkers to assess cell health during assisted reproduction.



## 1. Introduction

There is a need to quantify extracellular biomarkers such as reactive oxygen species (ROS),[1, 2] zinc ($Zn^{2+}$)[3-5] and pH,[6, 7] in assisted reproductive technologies to allow for the assessment of cell health. This has applications in assisted reproduction for both human and agriculturally important species. These external biomarkers must be identified non-invasively in order for the reproductive cells to be viable for subsequent use in reproductive technologies such as *in vitro* maturation (IVM),[8] *in vitro* fertilisation (IVF)[9] and embryo culture.[10] Embryos need to be grown in small volumes (10-100 µL) in order to prevent dilution of growth factors these embryos produce to support their own development.[11] Optical fibre-based fluorescent probes[12-15] provide access to these small volumes for the non-invasive detection of extracellular biomarkers, with reported examples measuring pH,[16-18] metal ions[19, 20] and ROS[21-23] in biological environments.

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Optical fibre-based fluorescent probes generally have a functionalised surface containing analyte-specific sensing molecules.[24] The proximal end of the fibre is connected to a light source and detector in order to facilitate light delivery and collection from the functionalised surface.[12] These optical fibre-based fluorescent probes have found use in a range of applications, where the fibre geometry (~100-200 µm diameter) provides access to micro-volumes of analyte in spatially hindered environments.[15] We recently reported a pH-sensitive optical fibre probe, referred to here as **OFP1**, and its measurent of extracellular surface pH in excised human breast cancer tissue samples. The functionalised surface of this probe contains the pH sensitive fluorophore 5(-6)-carboxynapthofluorescein (CNF) embedded within an acrylamide polymer coating on the tip of a 200 µm diameter multimodal optical fibre (see Figure 1).[18] **OFP1** successfully detected differences in extracellular acidity, which enabled the margins between healthy and cancerous breast tissue to be discerned.

Here we present a new application for **OFP1** measuring the pH in 5 µL drops of culture medium containing unfertilised eggs (oocytes) with their surrounding support cells (the cumulus), which is collectively known as the cumulus-oocyte-complex (COC). Prior to fertilisation, the oocyte needs to go through maturation to prepare its genetic material and cytoplasm for sperm entry and its transformation into an embryo.[25] During this time, the cumulus cells are metabolically active and communicate with the oocyte bidirectionally to produce the correct amount of energy needed by the oocyte.[26] A biproduct of this metabolism is lactic acid,[27, 28] which would likely acidify the immediate microenvironment around the COC. Previous work has shown that both oocyte and embryo metabolic activity is indicative of subsequent developmental capacity.[29] COCs were chosen for this study because of the ability to chemically control their speed of maturation,[30] thus influencing their metabolism and causing changes that could be detected by **OFP1**.

To the best of our knowledge, this work presents the first instance of local pH being used as an indirect measure of *in vitro* COC metabolism. Improved measurement precision was obtained compared to previous work, enabling 0.01 – 0.05 pH unit distinction between treatment groups in the culture media over a 7.30 – 7.45 pH range. Importantly, **OFP1** was shown to be cell-safe with no inherent chemical toxicity to embryos or light-induced phototoxicity to the COCs. This optical technology presents a promising platform for the measurement of pH and the detection of other extracellular biomarkers to assess cell health during assisted reproduction.

## 2. Experimental Design

### *2.1 Materials and Equipment*

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

*Optical Fibre Probe:* Multi-mode optical fibre (200 µm core diameter, FG200UCC; Thorlabs, Newton, NLJ, USA) was used to manufacture all **OFP1** probes.

*Microscope Setup:* A Nikon Eclipse TE2000-E inverse microscope (Nikon Instruments Inc., Melville, NY, USA) with a Nikon Digital Sight DS-U3 camera attached and Nikon NIS Elements Freeware v4 32 bit was used to visualise the probe and the COCs (e.g. Figure 1d). An Eppendorf TransferMan NK2 micromanipulator (Eppendorf, Hamburg, Germany) was used to position **OFP1** in the 5 µL drops of culture medium.

*Culture Medium Preparation:* Eagle's Minimum Essential Medium alpha formualtion (αMEM) was prepared from powder containing Earle's salts and non-essential amino acids and excluding L-glutamine, phenol red and sodium bicarbonate. Fresh sodium bicarbonate, gentamicin, glutamax and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic (HEPES) acid and salt were added to supplement the media and make it's pH bench stable without gas buffering respectively. Bovine Serum Albumin (BSA, MP Biomedicals, Solon OH USA, 4 mg/mL) was added fresh to the media immediately before use for each replicate.

*Calibration Standards:* Fresh αMEM was added in triplicate to separate Eppendorf tubes each containing 1 mL. Three tubes were set aside as base medium and all others were spiked with 0.001% v/v of either acid (0.1, 0.25, 0.5 and 0.75M HCl) or base (0.25 and 0.5M NaOH). Reference values for the pH of the calibration standards were obtained by recording the pH of the media standards, prepared in triplicate, at 37 °C with a glass electrode pH meter (InLab MicroPro-ISM electrode, Seven Excellence S400 pH meter, manufacturer stated accuracy ± 0.002 pH units, Mettler Toledo, Switzerland).

### *2.2 Optical Fibre Probe (**OFP1**)*

Silane solution was prepared from 2% (by volume) 3-(trimethoxysilyl)propyl methacrylate (TSPM) in pH 3.5 Milli Q water (adjusted with HCl) and ultrasonicated/vortexed (for up to 3 h) until the solution was completely clear. Note, it is imperative that the TSPM be relatively fresh (less than 6 months since opening). The free ends of connectorized multimodal optical fibres were then cleaved to expose a fresh surface for silanisation, and dipped into the silane solution for 75 min. The fluorophore polymerisation solution was prepared from (by weight) acrylamide (27%), bis(acrylamide) (3%; Polysciences, Warrington, PA, USA) and pH 6.5 PBS buffer (70%) with 0.4 mg/mL 5(-6)-carboxynapthofluorescein (CNF). Triethylamine (40 µL/mL) was added to the polymerisation solution immediately before use. The fibre ends were then rinsed in Milli Q water and immediately dipped into the fluorophore polymerisation solution. The input end of the optical fibres was sequentially coupled into a LED light source (LEDD1B, 405 nm, Thorlabs Inc., Newton, NJ, USA) and illuminated for 45 secs to photopolymerize the acrylamide polymer (encapsulating the CNF fluorophore) onto each fibre tip. Throughout this paper, a "scan" refers to a single 100 ms illumination of the tip to receive a fluorescence response from the CNF fluorophore.

## *2.3 Mouse Embryo Assay (MEA)*

A Mouse Embryo Assay (IVF VET Solutions, Adelaide, Australia) was conducted in order to assess the inherent chemical toxicity of the functional sensing end of **OFP1** towards mammalian embryo survival. Day 1 embryos were flushed from the oviduct of the mouse following mating. The probe was placed into the culture drop to expose the embryos to the test article for days 1-2 of culture. Early cleavage stage embryos were then moved to a subsequent culture medium drop in the absence of

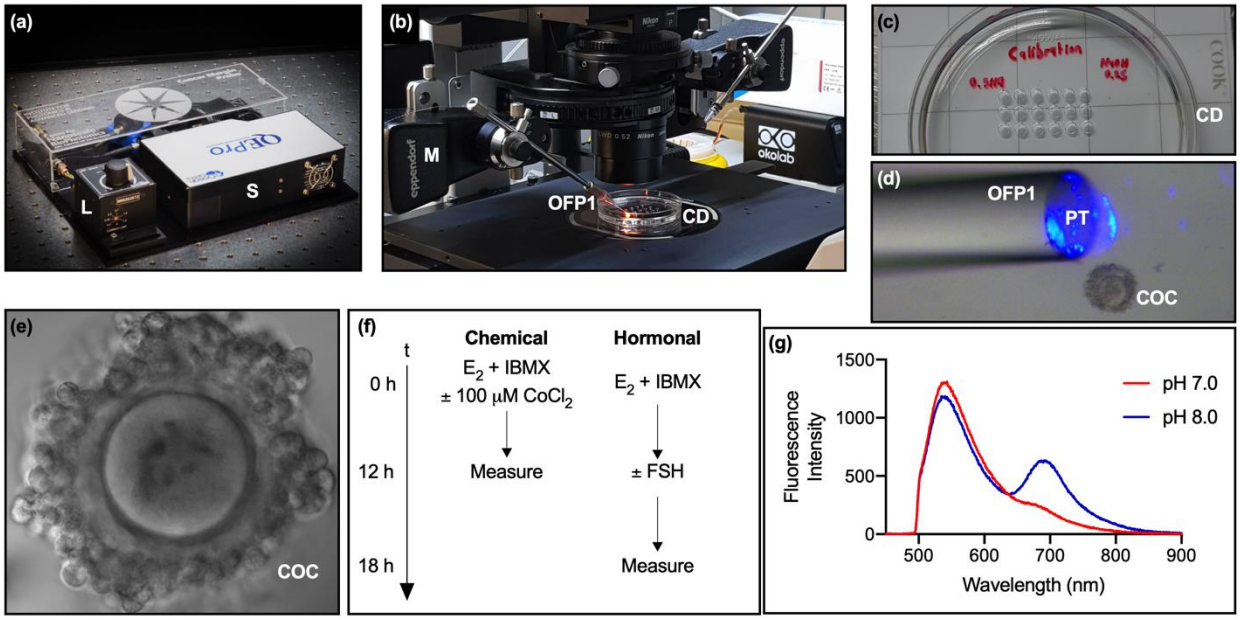


**Figure 1.** Diagram of the *in vitro* experimental procedure. (a) Portable spectrometer for measurement with **OFP1**; L = LED Light Source, S = Spectrometer. (b) Micromanipulator setup with **OFP1** fed through left arm into culture dish media droplet. The culture media petri dish (CD) is placed on a transparent glass which is a heating stage, allowing us to maintain the temperature of culture media within the culture dish at 37°C; M = micromanipulator, OFP1 = **OFP1** is fed through the micromanipulator arm. (c) Calibration culture dish with 5 µL drops of culture medium covered in paraffin oil. (d) **OFP1** probe, consisting of the optical fibre (OFP1) and polymer tip (PT), measuring pH in the microenvironment around the COC. (e) Ovaries were collected from eCG stimulated mice 46 h after injection and COCs were extracted and divided into treatment groups. (f) Chemical stimulation treatment groups were prepared by incubation of COCs with 0 µM or 100 µM $CoCl_2$, $E_2$ and IBMX for 12 h and subsequently measured. Hormonal stimulation treatment groups were prepared by incubation with $E_2$ and IBMX for 12 h, washed, and treated with 0.1 IU/mL FSH or plain media for an additional 6 h before measurement (18 h in total). (g) Example ratiometric fluorescence signals from **OFP1** used to determine pH comparing pH 7 (**red**) to pH 8 (**blue**).

protein to create a compromised culture environment for days 2-5 of culture in order to make any toxicity more apparent. Embryo development was recorded on day 5 of embryo culture, where there were >85% blastocysts developed, and thus the probe was deemed to be embryo safe according to industry standards.[31] The full certificate of analysis is detailed in the Supporting Information.

## *2.4* In vitro *Measurement Protocol*

*Experimental Setup:* The portable spectrometer setup (Figure 1a) was configured as described previously.[32] **OFP1** was fed through the left-side arm of an intracytoplasmic sperm injection micromanipulator (Figure 1b) to enable fine location control for insertion of the probe into 5 µL drops of culture medium (Figures 1c and 1d). Culture droplet temperature was maintained at 37°C by storage in a MINC bench-top incubator (COOK Medical, Bloomington, IN, USA) and through positioning on the microscope heating stage while scanning took place.

***OFP1*** *Pre-bleaching:* A set of "pre-bleaching" scans (~ 800-1000 scans at 1 scan/5s, LED excitation 473 nm) were performed in 5 µL drops of pH 7.4 base culture medium at the start of each experimental replicate with a new **OFP1** probe.

*Experimental Measurements:* Immediately after the pre-bleaching step, experimental measurements were then commenced with a delay of no longer than 2 mins between scans throughout the experiment. Calibration measurements were performed before, mid-way through and after each treatment group to monitor for any potential fluorescence signal drift.

## *2.5 Animals*

All experiments were approved by The University of Adelaide Animal Ethics Committee (M-2015-072) and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Pre-pubertal CBA x C57Bl/6 F1 hybrid mice (3 – 4 weeks old) at 9 – 11 g were housed within the Laboratory Animal Services (University of Adelaide, Australia) under controlled temperature, photoperiod (12h light: 12h dark) with water and feed *ad libitum.* Pre-pubertal female mice were super ovulated with 5 IU Equine chorionic gonadotropin (eCG; Folligon, Intervet, Boxmeer, Netherlands) administered intra peritoneal and 46 h later mice were culled via cervical dislocation. Immature COCs were extracted by puncturing the follicles in the ovaries and allocated to the randomized and blind treatment groups (Figure 1e).

## *2.6 Treatment Groups*

The two COC treatments (chemical and hormonal) were performed concurrently as outlined in Figure 1f. For the cobalt chloride ($CoCl_2$) treatment, COCs were incubated in individual 5 µL culture droplets containing 3-isobutyl-1-methylxanthine (IBMX, 50 µM in EtOH), estradiol ($E_2$; 100 nM in EtOH) and $CoCl_2$ (0 µM or 100 µM). Ten individual culture droplets, 5 µL each, were pipetted on a culture petri dish and were incubated for 12 h before pH measurement. Similarly, for the Follicle Stimulating Hormone (FSH; Puregon, Organon, Netherlands) treatment, COCs were incubated in IBMX (100 µM) and $E_2$ (100 nM) for 12 h as above (without $CoCl_2$) in order to maintain meiotic arrest.[33, 34] While the $CoCl_2$ group measurement began, the FSH grouped COCs were washed 3 times in plain handling media and transferred to either individual 5 µL culture medium droplets containing FSH (0.1 IU/ml) or no additives ('No FSH' group) for 6 h before pH measurement. In both cases, the chemical[35, 36] and hormonal[27, 28] treatments serve to stimulate COC lactic acid production through hypoxia and maturation respectively, inducing a local and potentially detectable pH change in the immediate microenvironment around the cells. All dishes were prepared with the culture medium droplets in a randomized order and covered with parrafin oil (Merck, Darmstadt, Germany) to prevent evaporation. Importantly, individual COCs were allocated to droplets in a randomized fashion and the identity of each droplet was blind to the researchers conducting the **OFP1** pH measurements.

## *2.7 COC Phototoxicity*

After each replicate of pH measurements, all treated COCs and incubator control COCs that were not exposed to **OFP1** were fixed in 400 µL of 4% paraformaldehyde in phosphate buffered saline (PBS; 1 tablet per 200 mL Milli-Q water). 1 h later embryos were transferred to solution of polyvinyl alcohol (PVA) in PBS (PVA-PBS solution: 0.3mg PVA/mL of PBS). Goat serum (Jackson Immuno, Philadelphia, PA, USA; 10 % in PVA-PBS) and anti-γH2AX primary antibody (Cell Signalling Technology, Danvers, MA, USA; 1:200 dilution with 10% goat serum in PBS-PVA) were added, and the embryos were incubated in this solution for 24 h at room temperature to avoid non-specific binding. COCs were then washed 3 times in PBS-PVA solution and incubated for 2 h with anti-rabbit Alexa Fluor 594-conjugated

secondary antibody (Life Technologies, Carlsbad, CA, USA; 1:500 dilution in 10% goat serum in PVA-PBS solution) and 6-diamidino-2-phenylindole (DAPI, 3 µM) at room temperature. COCs were then mounted onto a glass microscope slide with fluorescent mounting medium (Dako Inc., Carpinteria, CA, USA) and gently covered with a coverslip using a spacer (Thermo Fisher, Waltham, MA, USA). Fixed COCs were then imaged using an Olympus FV 3000 confocal microscope (Olympus, Tokyo, Japan). A negative control was performed via omission of the primary antibody from an otherwise complete reaction. Imaging took place in conjuction with COCs treated with 100 times higher $E_2$ than the standard experiment. This was done to induce stress and confirm the anti-γH2AX primary antibody was functional. Images were obtained with Laser Ex: 405nm, Emission Detection Wavelength: 430-470 nm for DAPI and Laser Excitation: 594nm, Emission Detection Wavelength: 610-710 nm for γH2AX positive cells using consistent exposure and gain settings.

### *2.8 Statistical Analysis*

All statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). pH values for each individual (blind, randomized) treatment group droplet were calculated by interpolation of the fluorescence signal from the average of two pH standard calibration lines, using the 'interpolation of a standard line' function in GraphPad Prism 8.0. Statistical analysis was performed to compare chemical and hormonal treatment groups (n = 10 in each treatment). Normality testing was first performed in order to determine whether parametric or non-parametric testing should be used. Statistical significance of the difference in the mean between the groups was evaluated using an Unpaired t-test for normally distributed data or Mann-Whitney test for non-normally distributed data. P-values were accepted as significant when less than 0.05. All calculated pH values are presented as Mean ± Standard Deviation (SD). It is important to note that experimental replicates are graphed individually due to different starting pH levels between different batches of culture media.

## 3. Results and Discussion

### *3.1* ***OFP1*** *Pre-bleaching and Positioning*

Initial repeated scans 5 s apart through freshly prepared **OFP1** probes revealed that the CNF response signal exponentially decays over time (Figure 2a). After approximately 600-1000 scans (~ 1-1.5 hours), depending on the size of the polymer tip, the signal reaches a plateau where continued measurement in the same conditions yields the same fluorescence response (see Figure 2b). This effect can be attributed to the photobleaching of CNF; a phenomenon by which photon excitation induces molecular damage of the fluorophore resulting in a decrease in the fluorescence emission intensity of the sample.[37] The effect of fluorescence photobleaching is known to be magnified in an optical fibre tip compared to bulk solutions.[16] The signal from a fibre tip has also been shown to follow a mathematically predictable decay,[38] eventually stabilising where the fluorophore population bleaches no further. By commencing experimental measurements after a period of "pre-bleaching", as detailed in *Section 2.4*, the initial exponential fluorescence signal decay of **OFP1** can be overcome until a consistent fluorescence signal is obtained. Therefore, any observed changes in fluorescence signal after this point may be attributed to meaningful pH changes in the medium being measured.

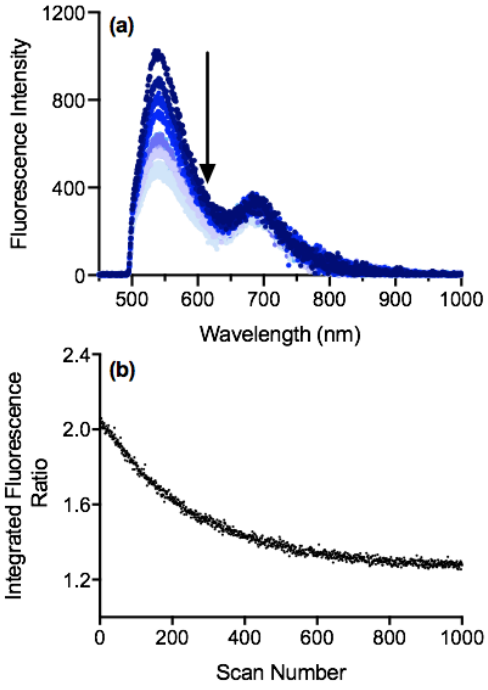


**Figure 2.** Fluorescence prebleaching of **OFP1** over 1000 scans 5 s apart. (a) Fluorescence emission spectra from **OFP1** at 5 minute intervals over the prebleaching period. (b) Relative fluorescence ratio signal follows a near exponential decay, reaching a signal plateau after approx. 1000 scans.

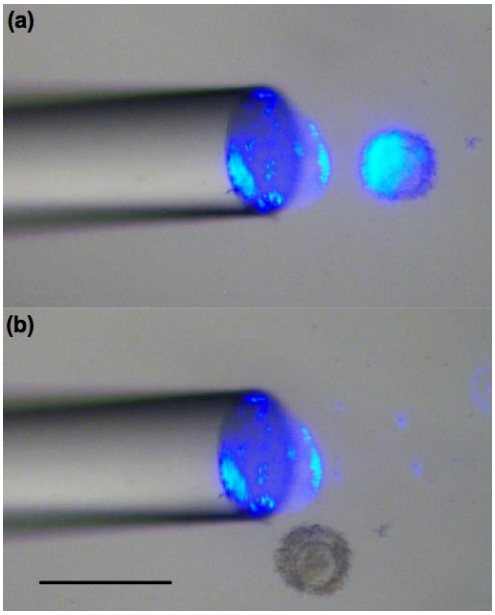


**Figure 3. OFP1** within a 5 µl media droplet, positioned (a) directly towards a COC with significant blue light irradiation of the cell and (b) above the COC with no blue light irradiation of the cell. The pH probe was positioned as shown in image (b) for all measurements. Scale bar denotes 200 µm.

With the necessary amount of pre-bleaching scans determined, the positioning of **OFP1** in spatial relation to COCs within a 5 µL drop of culture medium was next assessed. Fluorescence spectra recorded with the probe positioned directly at (Figure 3a) or slightly above (Figure 3b) the COC revealed near identical maxima and intensity in CNF response (Figure S1), indicating that the short 100 ms irradiation time with **OFP1** is not sufficient to induce an interfering level of cellular autofluorescence from native endogenous fluorescent species.[39, 40] However, all scans were performed directly above the COC (Figure 3b) to minimise the direct blue light exposure and hence the potential for these cells to experience phototoxicity.

### *3.2* ***OFP1*** *Phototoxicity*

Blue light has been previously shown to incur DNA double strand breaks in retinal cells,[41] mitochondrial DNA damge and increased ROS production in epithelial cells,[42] and, following a 10 min exposure of two cell embryos, increased rates of cell death and decreased blastocyst formation.[43] Therefore, it was important to confirm that indirect **OFP1** light exposure, as shown in Figure 3b, did not cause phototoxicity through blue light exposure.

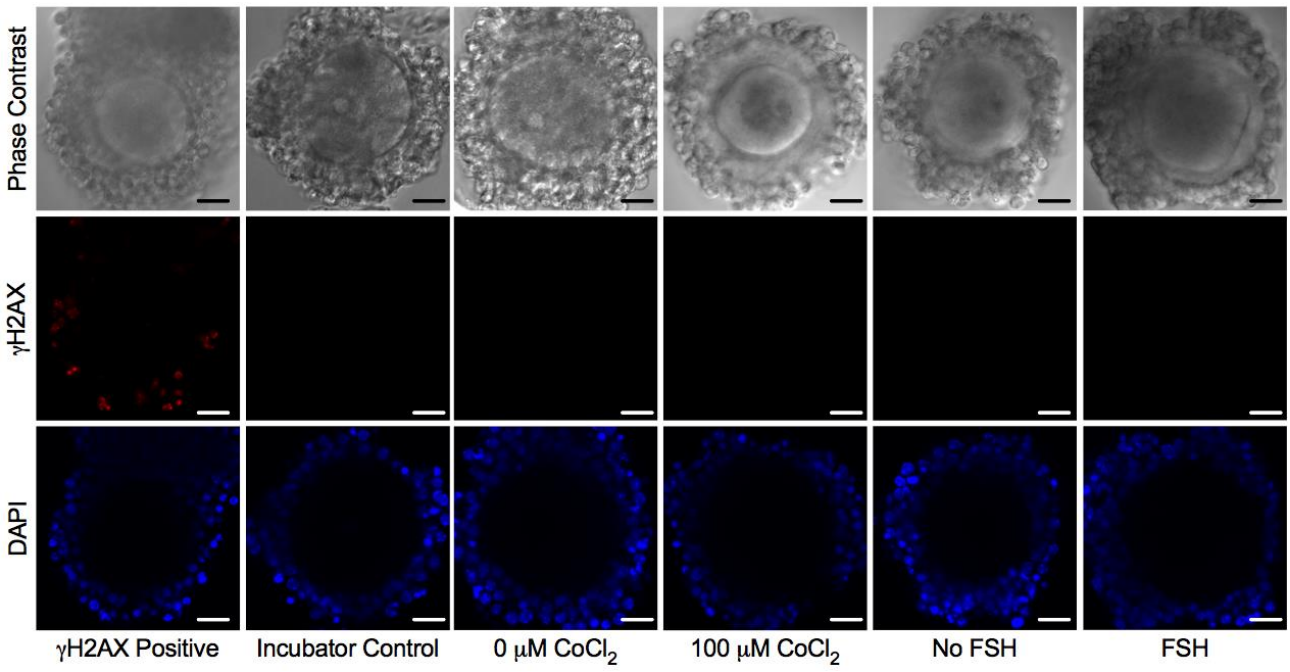


**Figure 4.** Immunohistochemical staining of fixed COCs following optical fibre light exposure. A γH2AX positive COC is included for reference compared to incubator control COCs that were not exposed to **OFP1** and the measured treatment groups 0μM $CoCl_2$, 100μM $CoCl_2$, no FSH and 0.1 IU/mL FSH. Scale bar represents 20 μm in all cases. DAPI blue stain is bound to all the nuclear DNA of the cumulus cells and γH2AX red stain is bound to any double strand breaks in the DNA, highlighting any sections of the DNA that are damaged. In all cases, no γH2AX staining was observed compared to the γH2AX positive COC, indicating no DNA damage was present in these cells.

In order to assess the genetic health of the cells, all scanned COCs were immediately fixed in paraformaldehyde and later stained to assess DNA damage. The prescence of this damage was established using γH2AX staining, a fluorescent antibody which binds to DNA double strand breaks.[44] The degree of damage can be quantified by costaining all DNA with the non-discriminant stain DAPI and comparing the fluorescent area of the two stains (data not shown). Confocal imaging of **OFP1**–exposed COCs from experimental replicates 1-4 showed no red γH2AX fluorescence in scanned COCs or unexposed incubator control compared to γH2AX positive COCs (Figure 4). Therefore no DNA double-strand breaks were induced by indirect light exposure from **OFP1** in the cumulus cells or oocytes (Figure 4). As hypothesised, the blue light of **OFP1** being directed away from the COC (Figure 3b) and relatively short irradiation time of 100 ms prevented any short-term damage to the cells. In addition to the MEA report (see Supporting Information), these results demonstrate that **OFP1** is not harmful to COC DNA integrity or preimplantation embryo development.

### *3.3 In vitro **OFP1** pH measurements*

The assessment of **OFP1** signal stability, positioning and toxicity means that the scans performed *in vitro* were both accurate and non-invasive to the cells. These experiments measured culture medium pH in the immediate microenvironment around COCs following chemical ($CoCl_2$) or hormonal (FSH) treatment of the cells, as detailed in *Section 2.6* and depicted in Figure 1e. Importantly, validation experiments with a conventional pH meter and **OFP1** showed that the treatments themselves did not directly induce a change in pH of the base αMEM in the absence of cells (Figure S2). Hence, the statistically significant changes observed *in vitro* can be wholly attributed to cell-induced acidification. Due to time constraints imposed by the necessary incubation times for the different treatments, the hormonal treatment groups always underwent measurement with **OFP1** 6 hours after the chemical treatment group in each experimental replicate, and hence the data is presented and discussed separately.

COCs treated with 100 µM $CoCl_2$ had a statistically lower calculated pH than untreated COCs in three of the four experimental replicates (Figure 5), and in all cases **OFP1** detected a 0.01 to 0.05 mean pH difference between the groups (Table S1). It is known that $CoCl_2$ acts as a hypoxia mimetic to induce lactic acid production,[35, 36] which is likely released into the medium immediately surrounding the COC. As $CoCl_2$ itself does not alter the medium's pH (Figure S2), the detected decrease in pH of these treated COCs is a result of a known cellular stress response. Therefore, these results indicate that **OFP1** is a suitable technology to detect pH differences in the microenvironment around a COC caused by cell behaviour.

Hormonal stimulation of COCs was next explored in order to investigate whether a biological event can trigger a measurable change in pH similar to the one caused by $CoCl_2$ exposure. In all three replicates, FSH stimulation resulted in a 0.02 to 0.03 mean pH unit decrease compared to untreated COCs (Figure 6, Table S1). As FSH increases lactic acid production by increasing the metabolic activity of cumulus cells,[27, 28] the difference detected here by **OFP1** is a direct result of a biologically significant process. Therefore, these results demonstrate that **OFP1** is sensitive enough to detect differences caused by a hormone that naturally alters the level of cumulus cell metabolism, which resulted in a detectable pH decrease in the microenvironment surrounding the COC.

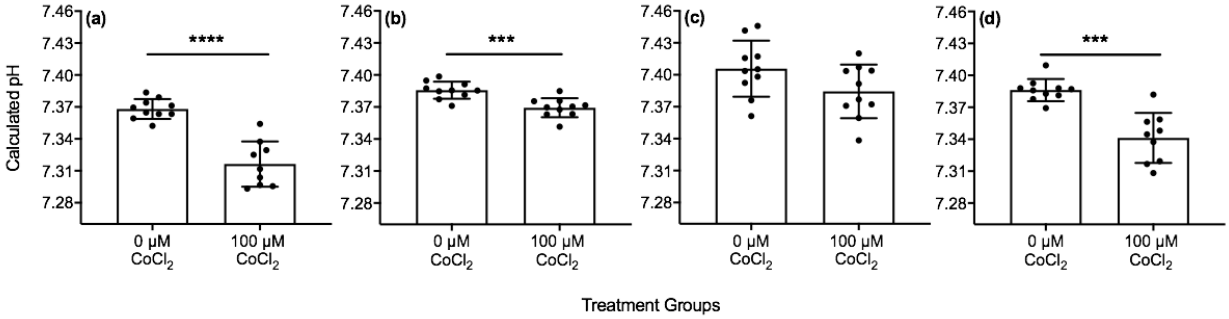


**Figure 5.** (a-d) Calculated pH values for cumulus oocyte complexes (COCs) exposed $CoCl_2$ (1 Replicate/graph; 10 COCs/group; Mean ± SD; Unpaired Student's t-test: a-c; Mann-Whitney test: d). Statistically significant differences in the mean calculated pH value between 0 µM-100 µM $CoCl_2$ COC treatment groups were observed across two replicates and repeated a fourth time to validate the non-significance of replicate three (****p<0.0001 and ***p<0.001).

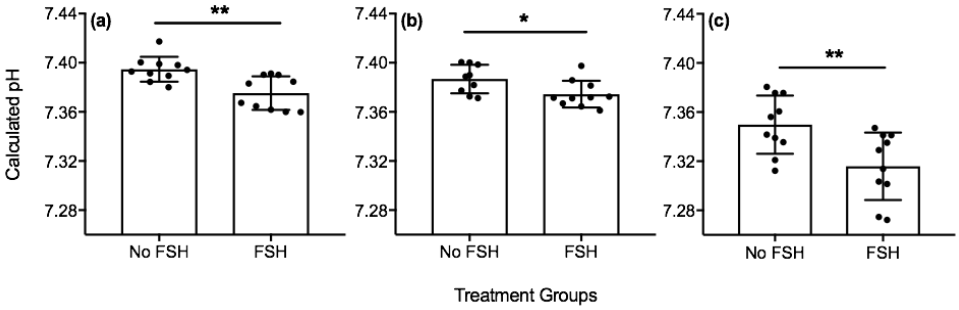


**Figure 6.** (a-c) Calculated pH values for cumulus oocyte complexes (COCs) exposed to ±FSH (1 Replicate/graph; 10 COCs/group; Mean ± SD; Unpaired Student's t-test: b, c; Mann-Whitney test: a). Statistically significant differences in the mean calculated pH value between No FSH and FSH COC treatment groups were consistently observed across three replicates (**p<0.01 and *p<0.05).

## 4. Conclusion

This work presents the first instance of local pH being used as an indirect measure of individual COC metabolism *in vitro*. Prebleaching the CNF fluorophore prior to each experiment in order to obtain a stable signalimproved **OFP1** measurement precision. This enabled the detection of meaningful pH changes in the microenvironments being measured. Obtaining these results was not influenced by cellular autofluorescence and did not have a toxic effect on COC health. Therefore, **OFP1** was both non-invasive and accurate enough to discriminate between groups 0.01 – 0.05 pH units different from one another. These differences can be wholly attributed to cell-induced acidification as the treatments without cells did not show significant differences in pH. **OFP1** was able to detect increased lactic acid production in the cumulus cells due to $CoCl_2$ induced hypoxia and FSH induced COC maturation. This indicates functionalised optical fibres are both appropriate for detecting subtle changes in the microenvironment surrounding the COC and sensitive enough to detect changes as a direct result of a biological event. There is great potential for this technology to be adapted to detect other biomarkers of growth and metabolism throughout *in vitro* embryo development for diagnostic purposes.

## Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's website.

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