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Advancing Carbon Nanomaterials-Based Device Manufacturing through Life Cycle Analysis, Risk Assessment and Mitigation

Saccharomyces cerevisiae (Budding Yeast)

Standard Operating Procedure Series: Toxicology (T)

Alan J. Kennedy, Chelsea Campbell, Cullen Horstmann,
and Kyoungtae Kim

June 2019

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Environmental Laboratory

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Final Report

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Abstract

Engineered Nano Materials (ENMs) are commercially used in everyday products, including zinc sunscreens and water resistant fabrics and surfaces, but in the future, they may be used in targeted treatment of cancer, printable monitoring systems, and foldable phones. Understanding the effects of ENMs on the environment is crucial for the responsible use of these technologies. The aim of this project is to develop a standard operating procedure (SOP) for investigating the effects of ENMs on budding yeast (*Saccharomyces cerevisiae*). The ENMs used to develop this protocol were Ag and CdSe/ZnS. Toxicity was determined using plate assays to analyze the effect of ENMs on the growth cycle, Fun-1 staining assays to understand the effects on cell metabolism, and quantitative reverse transcriptase-based polymerase chain reaction (PCR) and RNAseq to understand the effects on genetic expression. From plate assays, doubling times, average time spent in lag phase, maximum concentrations were determined and compared between yeast grown in varying concentrations of ENMs, and a yeast grown in a control environment. Fun-1 staining determines the amount of metabolically active cells present in a treated cell culture. RNAseq and Quantitative PCR results, in expression levels of genes, are used to determine potential toxic effects of ENMs.

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Preface

This study was conducted for project for Task 3: Environmental Health under PE 0603728A "Advancing Carbon Nanomaterials-Based Device Manufacturing through Life Cycle Analysis, Risk Assessment and Mitigation." This study was directed by Rishi J. Patel, Senior Research Scientist at Missouri State University's Jordan Valley Innovation Center. The technical monitor was Jerry Miller (CEERD-EL-EMJ).

The work was performed by the Environmental Risk Branch (EPR) of the Environmental Processes Division (EP), U.S. Army Engineer Research and Development Center, Environmental Laboratory (ERDC-EL). At the time of publication, Dr. William M. Nelson was Branch Chief (CEERD-EPR), Mr. Warren P. Lorentz was Division Chief (CEERD-EP), and Dr. Elizabeth A. Ferguson (CEERD-EMJ) was the Technical Director for Environmental Quality and Installations. The Deputy Director of ERDC-EL was Dr. Jack E. Davis and the Director was Dr. Ilker R. Adiguzel.

COL Ivan P. Beckman was Commander of ERDC, and Dr. David W. Pittman was the Director.

Unit Conversion Factors

Multiply	By	To Obtain
microinches	0.0254	micrometers
microns	1.0 E-06	meters
Nanometers (nm)	1.0 E -9	meters
ounces (mass)	0.02834952	kilograms
ounces (U.S. fluid)	2.957353 E-05	cubic meters
square inches	6.4516 E-04	square meters
square miles	2.589998 E+06	square meters
square yards	0.8361274	square meters

Acronyms and Abbreviations

cRNA	Complementary DNA
DI	Demineralized
DNA	Deoxyribonucleic Acid
DoD	Department of Defense
EL	Environmental Laboratory
ENMs	Engineered Nano Materials
ERDC	Engineer Research Development Center
g	grams
GNPs	Gold Nanoparticles
hr	Hours
HQUSACE	Headquarters, U.S. Army Corps of Engineers
L	Liters
LS	Low Sample Size
min	Minutes
mL	Milliliters
mRNA	Messenger RNA
µL	Microliters
NSP	Nanosilver Particles
OD	Optical Density
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
RNA	Ribonucleic Acid
ROX	6-carboxyl-X-Rhodamine

RPM	Revolutions Per Minute
SGD	Saccharomyces Genome Database
SD-Glu	Synthetic Defined Glucose
SOP	Standard Operation Procedure
TC	Test Concentrations
USACE	U.S. Army Corps of Engineers
USEPA	U.S. Environmental Protection Agency
YNB	Yeast Nitrogen Based
YPD	Yeast Peptone Dextrose

1 Introduction

The standard operating procedure (SOP) described herein for assessing the properties of nanotechnologies was developed under a public/private partnership between the U.S. Army Engineer Research and Development Center (ERDC) and the Jordan Valley Innovation Center and Brewer Science on "Advancing Carbon Nanomaterials-Based Device Manufacturing through Life Cycle Analysis, Risk Assessment and Mitigation."

This SOP describes how to determine the toxicity of Engineered Nano Materials (ENMs) using Saccharomyces cerevisiae (budding yeast) as the model. This SOP details the execution of growth assays and gene expression analysis via qPCR and RNAseq of yeast exposed to ENMs.

Budding yeast is a well-studied model organism for eukaryotic cells. This is because the cell structure is very similar to mammalian cells, therefore, understanding how ENMs effect yeast will allow for a better understanding of the potential impact they will have on other organisms.

1.1 Background

ENMs have great potential use in future science. The properties that make ENMs unique, and therefore valuable, are their small size, large surface area to volume ratio, aggregation, chemical composition, solubility, and shape (Nel et al. 2006). ENMs are currently used in over 1000 commercially available products, ranging from sunscreens to water resistant surfaces (Damoiseaux et al. 2011). In the future, ENMs have been predicted to play a part in targeted disease treatment, nano-robotics, and next generation electronics. The applications of ENMs is increasing almost daily, however, the understanding of their toxicity is lagging behind technology development, thus encumbering safe and rapid deployment of these materials. In order to responsibly use these technologies, understanding how ENMs interact with the environment is a necessity. Their size allows them to interact at a molecular level, making them potentially useful in many applications, however, it also makes them potentially dangerous.

Nearly every class of ENMs has been found to have some negative biological effect, the question is whether or not these effects pose a

significant health or environmental risk (Kwolek-Mirek and Zadreg-Tecza 2014; Colvin 2003). In order to determine whether a particular ENM is toxic or not, the specific properties of the materials being investigated, including size, shape, etc. must be considered. Studies have shown that even between batches of ENMs, toxicity can change. Many studies in academia and industry have attempted to develop methods of understanding the toxicity of ENMs. Gold nanoparticles (GNPs) have gained considerable attention for potential application in cancer treatment such as photothermal therapy (Riley and Day 2017) and it was found that GNPs of different sizes are not inherently toxic to human cells, including keratinocytes and leukemia (Connor et al. 2005; Patra et al. 2005). However, two nanometer (nm) GNPs functionalized with both cataionic, and anionic surface groups, were toxic (Goodman et al. 2004). Another metal nanomaterial that has been widely used in a range of biomedical applications, including diagnosis, treatment, drug delivery, and medical device coating is nanosilver particles (NSPs) (Ge et al. 2014). While it has been well-known that NSPs hold antibacterial (Kim et al. 2007), antifungal (Kim et al. 2008), antiviral (Sun et al. 2005), and anti-inflammatory (Nadworny et al. 2008), there exists a report demonstrating that NSPs at 100 μg/ml with different sizes in HaCat cells are not toxic (Ray et al. 2009). Although the mechanism underlying their toxicity on different cells and organisms is not yet fully understood, the general consensus is that NSPs cause cell membrane disruption (Sondi and Salopek-Sondi 2004; Kim et al. 2009), and oxidative stress (Nogueira et al. 2014).

Though animal studies using laboratory mice, rats, hamsters, guinea pigs, and rabbits would be ideal to determine the effects of a spectrum of ENMs, because of the speed at which new materials are being developed, animal subjects simply are not reasonable for high through-put testing. Budding yeast is a well-studied model organism for eukaryotic cells. It is inexpensive, easy to work, and has a short observable generation time. The cell structure and organization is very similar to that of other eukaryotes, and is often used as a model to understand pathways in mammalian cells. Methods of evaluating toxicity of environmental factors such as hydrogen peroxide, allyl alcohol, menadione, HfO_2, SiO_2, Al_2O_3, and CeO_2 in yeast are reported (Kwolek-Mirek and Zadreg-Tecza 2014; Garcia-Saucedo et al. 2011), but SOPs for assessing the effect of NSPs in yeast has not been documented. Therefore, this SOP that describes methods for determining toxicity of NSPs in yeast was developed. If it is possible to understand how the materials are effecting the yeast, it can be extrapolated how they may

affect animal health and the environment. In addition, this SOP will allow for the assessment of the effect of other ENMs in yeast.

1.2 Objectives

This SOP describes methods for determining toxicity of engineered nanomaterials in yeast.

1.3 Approach

Three main methods, including cell growth assay, RNAseq, and RT qPCR, are used to assess the effects of engineered nanomaterials on living organisms.

1.4 Scope

This SOP is developed to assess the impact of freely dispersed 20 nm NSPs in an aqueous culture media on *Saccharomyces cerevisiae* (budding yeast).

2 Terminology

2.1 Related documents

- ELX808 Absorbance Microplate Reader (BioTek).
- Probes for Yeast viability (Molecular Probes).
- RiboPure™ RNA Purification Kit, yeast (ThermoFisher Sci.).
- Qubit assay (ThermoFisher Sci.).
- TruSeq® Stranded mRNA Sample Preparation (Illumina).
- GoTaq® qPCR Master Mix for Dye-Based Detection Kit (Promega).
- Verso cDNA Synthesis Kit (Thermofisher Sci.).

2.2 Definitions

- DNA, deoxyribonucleic acid, the hereditary nucleic acid found in living organisms.
- RNA, ribonucleic acid involved in production of proteins.
- mRNA, a type of RNA that carries genetic information from DNA.
- cDNA, complementary DNA that is produced from mRNA.
- nm, the nanometer is a unit of length, equal to one billionth of a meter.
- Agglomerate, n—in nanotechnology, an assembly of particles held together by relatively weak forces (e.g., Van der Waals or capillary), that may break apart into smaller particles upon processing.
- Blank, n—solution containing everything but the component being measured.
- Vortex, v—using a vortex mixer, a vortex swirl is created in a liquid solution when pressed to the vortex mixer, thoroughly mixing the liquid.
- Dilution, n—the action of making a solution weaker in force, content, or value.
- Gene, — a distinct sequence of nucleotides forming part of a chromosome, the order of which determines the order of monomers in a polypeptide or nucleic acid molecule which a cell (or virus) may synthesize.
- Buffer, n— a solution that can maintain a nearly constant pH if it is diluted, or if relatively small amounts of strong acids or bases are added.
- Gram, a metric unit of mass equal to one thousandth if a kilogram.
- Hour, a unit of time equal to 3,600 seconds.
- ng, nanogram is a metric unit equal to one billionth of a gram.

- Optical density, measurement of absorbance value of the culture medium used in an experiment.
- PCR, polymerase chain reaction is a technique to amplify a few copies of DNA, generating larger quantities of a specific target DNA.
- Q.S., Latin phrase meaning “a sufficient quality.”
- Supernatant, a — denoting the liquid lying above a solid residue after crystallization, precipitation, centrifugation, or other process.
- Vitality, — the capacity to live, grow, or develop
- Viability, — alive; capable of living, developing, or reproducing
- Centrifuge, —a machine with a rapidly rotating container that applies centrifugal force to its contents, typically to separate fluids of different densities (e.g., cream from milk) or liquids from solids.
- Aqueous phase — the water portion of a system consisting of two liquid phases, one mainly water, the other a liquid immiscible with water.

3 Materials and Apparatus

3.1 Materials

- Distilled Water
- Petri dishes
- RNase and DNase free filter pipette tips
- Round bottom 96 well plate
- Culture tubes
- Spectrophotometry cuvettes
- Personal Protective Equipment (PPE) - gloves, lab coat, goggles, etc.
- Ethanol
- GoTaq® qPCR master mix for dye-based detection
- Verso cDNA synthesis kit
- Qubit Assay kit (Fisher Scientific)
- Probes for Yeast viability kit (Molecular Probes)
- RiboPure™ RNA purification kit
- TruSeq® Stranded mRNA sample preparation kit
- YNB (Yeast Nitrogen Base)
- ROX (6-carboxyl-X-Rhodamine) used as passive reference dye
- SYBR Green used as a nucleic acid stain

3.2 Equipment/Apparatus

- GEN 5: Software needed to run ELx808 absorbance microplate reader.
- ELX808 Absorbance Microplate Reader (BioTek): to measure cell growth.
- Biomate 3 Spectrophotometer (ThermoFisher Sci.): For assessing optical density of samples.
- Qubit 3.5 Fluorometer: to measure deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) concentration.
- Incubator: for growing yeast plates.
- Shaker Incubator: for growing liquid yeast cultures.
- pH meter: for testing the pH of buffers.
- Real-Time PCR System.
- Olympus IX81 motorized inverted microscope with fluorescence and DIC optics.
- Vortex mixer.
- Heat block.

4 Procedure

- First, analyze cell viability via growth assay of liquid medium cultured yeast in presence of varying concentrations of ENMs using EL x 808 plate reader.
- Then, analyze the resulting data to determine the effect of the ENMs on cell growth cycle.
- Determine the lowest concentration of ENMs at which there is an inhibitory effect.
- Treat cells with the determined concentration of ENMs and stain them with FUN-1 dye to access cell vitality by determining the metabolic activity of the cell.
- Extract mRNA from cells treated with the determined concentration of ENMs and from the control untreated cells.
- Then convert messenger RNA (mRNA) to complementary DNA (cDNA).
  a. Send cDNA out to be sequenced.
  b. Then analyze the sequences to determine any significant changes in expression levels between the cells treated with ENMs and control untreated cells.

and

  c. Using qPCR, analyze the expression levels of select genes (from RNAseq) to assess whether gene expression levels change as a result of toxic effects of ENM exposure.

4.1 Specimen preparation

4.1.1 Preparation of media

- Synthetic Defined Glucose media (SD-Glu)
  a. 3.4 g yeast nitrogen based (YNB) in 500ul DI Water
  b. 20 g Glucose
  c. 5 g Ammonium Sulfate
  d. Q.S. with demineralized (DI) water to 1000ml
- 2x Synthetic Defined Glucose (2x SD Glu) media
  a. Same recipe as above but Q.S. to 500ml.
- Yeast Peptone Dextrose (YPD) solid media
  a. 20 g Glucose in 500 µl DI Water
  b. 20 g Peptone
  c. 20 g Agar
  d. 10 g Yeast Extract

e. Q.S. to 1000 ml with water

- Yeast Peptone Dextrose (YPD) liquid media
  a. Same recipe as above without addition of Agar

4.1.2 Preparation of cells

- Fresh yeast (Strain name: S288C) culture is made by streaking on YPD plate from -80 °C stock S288C cells. Grow plate over two days in incubator set at 30 °C.
- From the plate a fresh liquid culture is made by inoculating one colony from the plate into 3 mL of SD-Glu minimal media and grown over night in 30 °C shaker incubator. This culture should be used as liquid cell stock and stored in 4 °C.
- Before each test, a new culture is inoculated by taking ~10 µl from the previously made liquid stock, and pipetting it into fresh SD-Glu and grown over night in 30 °C shaker incubator.

4.1.3 Sample Preparation for RNA extraction

- Prepare yeast culture (S288C) and grow them to reach mid-log phase Optical Density (O.D) of 0.3–0.6 in a spectrophotometer).
- Treat the desired concentration of silver during the continuous culture of the mid-log phase cells for five hours. This should be in a replicate of at least four times.
- The same is done for the three control samples, with media only.

4.2 Viability screening

4.2.1 Plate reader set-up

- Using Gen 5 software to control the Elx808 plate reader.
- A new protocol should be created. The procedure should be made as follows:
  a. Run Time: 24 hours (hr), Interval: Every 10 minutes (min)
  b. Kinetic Absorbance 594
  c. Shake: Fast, Continuously
- Using the Plate layout function, the location and identification of each test and sample should be defined.
- Using the Power Export Builder, the structure of the exported Excel file can be preset.

4.2.2 96-Well plate set-up

- A fresh culture of S288C cells is made the night before in SD-Glu minimal media.
  a. Refer to supplementary Figure 1 in the supplemental section. This is a visual guide of how the plate should be set up.
- Desired test concentrations (TC) of nanoparticles should be chosen, depending on the concentration of the stock. The highest TC should not exceed half the stock concentration. The concentrations should range down to 0.5µg/mL or lower. The rest of the TC should represent a series of concentrations between the highest and lowest chosen.
  a. (i.e., for silver testing with a stock of 20µg/ml the tested concentrations were 10µg/ml, 5µg/ml, 2µg/ml, 1µg/ml, 0.5µg/ml, 0.1µg/ml, and 0.05µg/ml).
- From the silver stock, 2x test concentration (2xTC) dilutions should be made into fresh test tubes. The dilution should be done with SD-Glu buffer. The final volume of each dilution tube of silver should be 900µl. Each tube should be vortexed to mix.
- Each column on the 96-well plate represents a different test concentration, with the last column used representing the control, this will be treated with buffer containing no ENMs
- The 96-well plate is designed so there will be four treated wells and four blank wells for each concentration including the control.
- Starting with the first column, this should represent the highest chosen concentration. 100µl of the highest 2xTC, created in step 3, should be pipetted into each of the eight wells in the first column. Moving to the next column, this should then be repeated for the second highest 2xTC. This should be completed for each of the remaining 2xTCs and columns.
- In the control well, 100µl of SD-Glu buffer should be added to each well.
- Pipette 100µl of 2x SD-Glu buffer into each blank well (four for each TC).
- Remove the fresh culture prepared 24hr ago from the incubator and determine its optical density using the spectrophotometer. The OD is used as a representative of cell concentration in the media.
- Using the overnight culture, create in a fresh tube a dilution that is 2x the desired test OD using 2xSD-Glu, at a volume high enough to dispense 100µl into each test well.
- 100µl of the diluted cells in 2xSD-Glu are pipetted into each test well.
- The plate is then placed into the ELx808 plate reader and run as a new experiment using the previously created protocol.
- Once the run has completed the data should be exported to Excel and saved.

4.3 Vitality screening

FUN-1 vitality screening

- Prepare 0.2 μm filtered water containing 10mM Na-HEPES containing 2%D-(+)-glucose (pH 7.2)
- Prepare a fresh overnight culture in 3 mL SD-Glu media the night before preforming this assay, from a fresh culture prepared from stock the day before.
- Create a 3 mL culture at an optical density at 600nm of 0.15 in SD-Glu media with the desired test ENM concentration. Let this grow in the shaker incubator for five hr.
- Remove the treated culture from the incubator.
- Take 300 μL of the treated cell culture and add it to a micro centrifuge tube containing 1 mL of the sterile 0.2 μm filtered water containing 2%D-(+)-glucose and 10 mM Na-HEPES.
- Concentrate by centrifugation for five min at 10,000 x g.
- Remove the supernatant and re-suspended the pellet in 1mL of the sterile 0.2 μm filtered water containing 2%D-(+)-glucose and 10 mM Na-HEPES
- Into the same tube from step 7, add 300 μL more of the treated culture from step 4, and repeat steps 6 and 7.
- Repeat step 8 once more so that a total of 900μL has been removed from the original culture, centrifuged, and is now suspended in 1 mL of sterile 0.2 μm filtered water containing 2%D-(+)-glucose and 10 mM Na-HEPES.
- Add 1 μL of 10 μM FUN-1 to the yeast cell suspension from step 9 and vortex to mix. Cover the micro centrifuge tube completely with tape to block out all light and incubate in a shaker incubator at 30°C for 30 min.
- Remove the micro centrifuge tube from the shaker incubator and remove the tape.
- Centrifuge the mixture at 3000 revolutions per minute (rpm) for two minutes to wash, remove the supernatant.
- Re-suspend the pellet in 1 mL of sterile 0.2 μm filtered water containing 2%D-(+)-glucose and 10 mM Na-HEPES.
- Repeat steps 12 and 13.
- Perform step 12 once more, then re-suspend the pellet in 25 μL of sterile 0.2 μm filtered water containing 2%D-(+)-glucose and 10 mM Na-HEPES.
- Take 2.5 μL of the mixture and mount it on a glass slide.
- Visualize the cells on Olympus IX81 motorized Inverted Microscope with fluorescence and DIC optics, on the conventional setting, using both red and green fluorescence.

- Approx. 20 images should be taken of each test concentration. A control sample with no ENM exposure should be tested along with each test.

4.4 RNA extraction

Using samples prepared as specified in 4.1.4, the extraction should be performed using the protocol and materials from RiboPure™ RNA Purification Kit (ThermoFisher), yeast. Below are the steps of the RiboPure™ RNA Purification Kit protocol, each step is described in further detail in the protocol.

4.4.1 Cell disruption and initial RNA purification

- Dispense 750 µL zirconia Beads into a 1.5 mL screw cap tube for each sample
- Collect up to 3 x 108 cells by centrifugation.
- Re-suspend cells in lysis reagents.
- Add mixture to Zirconia beads.
- Beat cells for 10 min using a bead beater in 2 min increments.
- Centrifuge for 5 min at room temperature, then transfer the aqueous phase to a fresh 4–15 mL tube.

4.4.2 Final RNA purification

- Preheat Elution Solution to 95 °C.
- Add 1.25 mL of 100% ethanol to each sample.
- Draw sample through a filter cartridge.
- Wash filter with 700 µL wash solution 1.
- Wash filter with 2 x 500 µL wash solution 2/3.
- Centrifuge for 1 min to remove excess wash solution from the filter.
- Elute total RNA in 2 x 50 µL preheated Elution solution.

4.4.3 DNase I Treatment

- Assemble the DNase digestion reaction.
- Incubate for 30 minutes at 37 °C.
- Treat with 0.1 volume DNase Inactivation reagent.
  a. Note: Samples can be stored at -80 °C for longer than two months.

4.4.4 RNA quantification

- The concentration of isolated total RNA samples was determined using a Nanophotometer (Implen) or Qubit 3.0 Fluorometer (ThermoFisher).

4.5 cDNA conversion and sequencing

4.5.1 mRNA isolation and conversion to cDNA

The cDNA library is constructed from the isolated RNA in 6.2.3 using the TruSeq® Stranded mRNA Sample Preparation Kit from Illumina Corporation. The Low Sample Size (LS) protocol of the Illumina TruSeq® Stranded mRNA Sample Preparation guide should be followed.

The major steps of this protocol are as follows:

- Purify and fragment mRNA molecules.
- Reverse transcribe the mRNA molecules to the first cDNA strand.
- Synthesize the second strand of the cDNA using uridine nucleotides and ligate adapters.
- Degrade the second strand of cDNA and PCR amplify the remaining first strand.
- Validate, normalize and pool the libraries. For this step, send cDNA samples to DNA sequencing centers (e.g., in this study, samples were submitted to Kansas Medical Genome sequencing center, Missouri University Core DNA Facility, etc.).

4.5.2 cDNA Sequencing

Sequencing of the cDNA libraries can be performed on an Illumina HiSeq 2500 Sequencing System (Kansas Medical Genome center). Sequencing 100 nucleotides from only one end of each sequence (single-end sequencing) was completed with cDNA libraries originated from three control and three NSP-treated cells. Briefly, cDNA libraries were applied into a flow cell and allowed to hybridize to the flow cell surface. The bound DNA fragments are subjected to amplification via a polymerase chain reaction, thus forming clonal clusters. These clusters are incubated with sequencing reagents that contain fluorescently labeled nucleotides, which allows addition of the first fluorescent base. The fluorescence originated from each cluster will be recorded, and a fluorescence detector recognizes the base. This cycle is repeated n times to create a read length of n bases.

The digitalized DNA sequences for all clusters are the outcome of the sequencing.

4.6 6 Real-time quantitative polymerase chain reaction (PCR)

To validate the differential gene expression data obtained from RNAseq results, a reverse transcription PCR method can be used. The isolated total RNA in 4.4.4 is used to produce cDNA library using Verso cDNA conversion kit (Fisher Scientific). A total of 1000–2000 ng of total RNA for each RNA sample was used for this process. A typical amount of cDNA produced from this conversion assay is around 50–100 ng/µl. The concentration of cDNA can be measured by a typical spectrophotometer or the Qubit Assay kit (Fisher Scientic).

The cDNA libray is used to amplify ALG9, FAF1, and SDA1 DNA sequences. ALG9, a house keeping gene, is selected as the reference gene due to that its expression in the presence of silver nanoparticle is not fluctuated based on the RNAseq results, whereas, FAF1 and SDA1 genes are highly elevated upon treatment of NSP

3 pairs of DNA primers used for the PCR are as follows:

Forward primer for ALG9 (CATTTGCTGTGATTGTCACTGACAG).

Reverse primer for ALG9 (TGATGCCCATAATGGCCATAATCTC).

Forward primers for FAF1 (CAGAGTATGGGTGGTGGAAATG).

Reverse primer for FAF1 (GATGACCATTCACCCTTGAGAG).

Forward primer for SDA1 (CCAGTACACTAACAGGCCCTATC).

Reverse primer for SDA1 (CTTACCGCGTCTACTACCAAAT).

Promega GoTaq qPCR Master mix (2x), water, 50 ng of cDNA template, and forward and reverse primers were mixed, then the mixture was added to the 96-well plate wells. No Mastermix control, and no cDNA control lacking 2x Mastermix, and cDNA, respectively were prepared as negative controls.

The 96-well containing all PCR mixtures was inserted into the Stratagene Mx3005p PCR machine. The PCR program was set up using MxPro QPCR software (https://www.thermofisher.com/us/en/home/brands/product-brand/sybr.html) to detect SYBR during PCR reaction and to select the ROX (6-carboxyl-X-Rhodamine) channel as the reference dye.

A standard two-step (denaturation at 95 °C and annealing/extension at 60 °C) 40 cycle PCR was performed to amplification of desired DNA.

5 Reporting

5.1 Analysis of results

5.1.1 Plate assay analysis

The growth plate analysis results in an Excel file containing optical density readings at 595 nm for each well. Each plate contains four test conditions containing cells, and four blanks (containing no cells, only media and ENMs).

- For each growth curve analysis, three plates should be run. The results should be analyzed separately and then averaged between the three.
- The OD should be corrected to reflect cell growth by subtracting the values of the blank wells from each test well OD.
- These corrected OD data in an Excel file are then used to determine the time spent in lag phase, maximum optical density at 595 nm, and doubling times for each test condition is determined.

5.1.2 FUN-1 metabolic activity analysis

- 20 images of each test condition and a control should be taken. This experiment should be replicated three times for each condition.
- Each cell in each image should be counted and recorded. The phenotype of each cell should be recorded. A cell glowing bright green with no visible red puncta is considered metabolically inactive and dead. Cells with red puncta are considered metabolically active.

5.1.3 Differential gene expression analysis: RNAseq

In order to determine the genes that are differentially expressed in response to ENM exposure, differential gene expression analysis must be performed on the sequences. There are several open (free) RNAseq analysis sources available for analyzing gene expression patterns, including ArrayExpressHTS, BioWardrobe, Chipster, easyRNAseq, ExpressionPlot, FX, and Galaxy. Among these, Galaxy is one of the most convenient platforms. This platform can be accessed via UseGalaxy.org, where a new account should be created if one is not active. For gene expression analysis, three control (non-treated) and three silver nanoparticle treated samples were sequenced. The following steps detail how Galaxy was used to perform this gene expression analysis.

- Download raw RNA-seq sequencing read data from the server of the sequencing provider using SQL database.
- The files should be uploaded to Galaxy using *Tools>Get Data* in the left hand menu. Upload each sequence file into Galaxy, specifying the reference genome to be S288C.
- The files should then be concatenated to merge them into one file. *Tools>Text Manipulation>Concatenate Dataset*.
- A quality check should then be performed on the files using *Tools>NGS:QC and manipulation> FastQC:Read QC*. This step will result in both a raw and webpage output. Looking at the webpage output, it will contain a series of charts and diagrams indicating quality assessments of the sequence.
- Send the concatenated files through *Tools>NGS:QC and manipulation> FastQ Groomer* to standardize the files into Sanger format.
- The groomed files should then be trimmed based on quality using *NGS:QC and manipulation>FASTQ Quality Trimmer*. Reads of zero length should be discarded, both the 5' and 3' ends should be trimmed with the window and step size left at the default value of 1. The minimum quality score should be set to >=20, this will result in removal of any base with a quality score lower than 20.
- *Tools>NGS:QC and manipulation > Compute quality statistics*, followed by *Tools>NGS:QC and manipulation > Draw nucleotides distribution chart* can be run on the FASTQ file generated in the previous step. This will generate a chart that shows the distribution of each base (A, T, G, or C) for each base position (1, 2, 3, 4, etc.) in the reads.
- Ideally the distribution of bases at each position should be equal, to achieve this, *Tools>NGS:QS and manipulation > FASTQ Trimmer* should be used to trim off bases at the 5' with unequal base distribution. Using the nucleotide distribution chart created in the previous step, the number of bases at the 5' end to be trimmed should be determined and entered as an absolute value. In this step, up to 15 bases from the 5' end should be removed.
- The double trimmed files should then be filtered based on length using *Tools>NGS:QC and manipulation> Filter FastQ*. Set the minimum size to 80, and the minimum quality to 20. No maximum size or quality should be set. This will result in all reads below the designated lengths being removed.
- In Galaxy, *TopHat* is then used to align the reads of the double trimmed filtered files to the reference genome. *Tools>NGS:RNA Analysis>TopHat*.

- *Cufflinks* is then used to assemble the transcriptome. *Tools>NGS:RNA Analysis> Cufflinks.*
- Steps 1–11 should be completed for each test condition and replicate. This may be done simultaneously via the use of a *Workflow*.
- To compare expression rates between conditions, *Cuffdiff* is used, via *Tools> NGS:RNA Analysis> Cuffdiff*. *Transcripts* should be the gene annotation file from the reference genome. As many conditions as were run, should be entered, with data from replicates being entered under the *Replicates* tabs, the input should be the *Accepted Hits* dataset output from step 10.
- *Transcript differential expression testing* dataset will be a table containing differential expression information for each gene identified between each condition. This file should be run through *Tools>Filter and Sort>Filter* to filter out any genes that are not significantly differentially expressed. The resulting file should be exported to excel.
- In Excel, apply Bonferroni correction or false discovery rate to control the number of genes that are false positives (Type I error) for responding to ENM.
- The resulting list of genes can be sorted based on their expression change. Genes with a fold change greater than 5 for either up or down regulation are considered to be the most dramatically differentially regulated. The study of the effects of differentially regulated genes in cells is beyond the scope of this SOP.

5.1.4 Real time quantitative PCR

To quantitate expression changes in FAF1 and SDA1 genes in NSP-treated samples, compared with non-treated samples, the Pfaffl method, a relative quantification tool (Pfaffl 2001), was used (Equation 1). In this method, the ALG9 gene was used as the reference gene to determine the relative quantitation of FAF1 and SDA1.

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (control-treated)}}}{(E_{\text{ref}})^{\Delta Ct \text{ ref (control-treated)}}} \quad \text{Equation 1}$$

Eref - the real-time PCR efficiency of ALG9 (theoretically 2, but EALG9 was 1.7 based on the serial dilution assay of ALG9). The serial dilution assay was repeated three times and the mean E value was determined.

Etarget - the real-time PCR efficiency of target gene such as FAF1 and SDA1. EFAF1 and ESDA1 was 1.71 and 1.69, respectively.

Ct values - cycle number at which detectable fluoresnce signal is achieved.

ΔCt value - Ct value from control samples – Ct value from silver nanoparticle-treated samples.

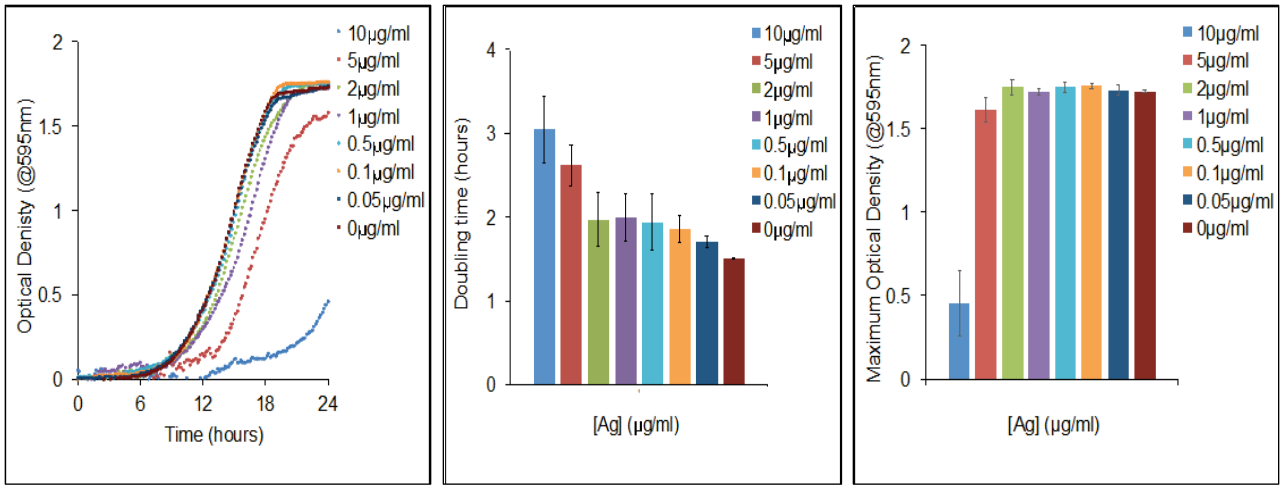
As shown in the equation, the ratio was calculated to determine fold change in gene expression.

5.2 Key results provided

5.2.1 Plate assays

The analysis of growth plate assays result in three key results for each of the following tested concentrations: Representative growth curves, Doubling Time, and Maximum Optical Density at 595nm reached. Figure 1 is an example of these results when testing Ag ENMs.

Figure 1. A. Growth curves of wild type (S288C) cells grown in SD Glu media containing differing concentrations (0 µg/ml-10 µg/ml) of silver nanoparticles. Each curve was produced by measuring the OD at 595 nm of the solution once every 10 min for 24 hr. Each point on the curve is an average of eight experiments. B. The final OD at 595 nm of each test concentration of silver nanoparticles, with the background subtracted. C. Doubling time of each concentration of silver nanoparticles. Found using the natural log of growth curves from (A) to determine the growth rate, which was then used to calculate the doubling time.



A B C

5.2.2 FUN-1

Key results from this assay are percentages of metabolically active cells present in each culture. Figures 2 and 3 are examples of these results when testing GNPs.

Figure 2. Percentage of cells metabolically active cells per cell culture treated with 5µg/ml, 10µg/ml, and control. The was no statistical difference found between test conditions in the percentages of metabolically active cells.

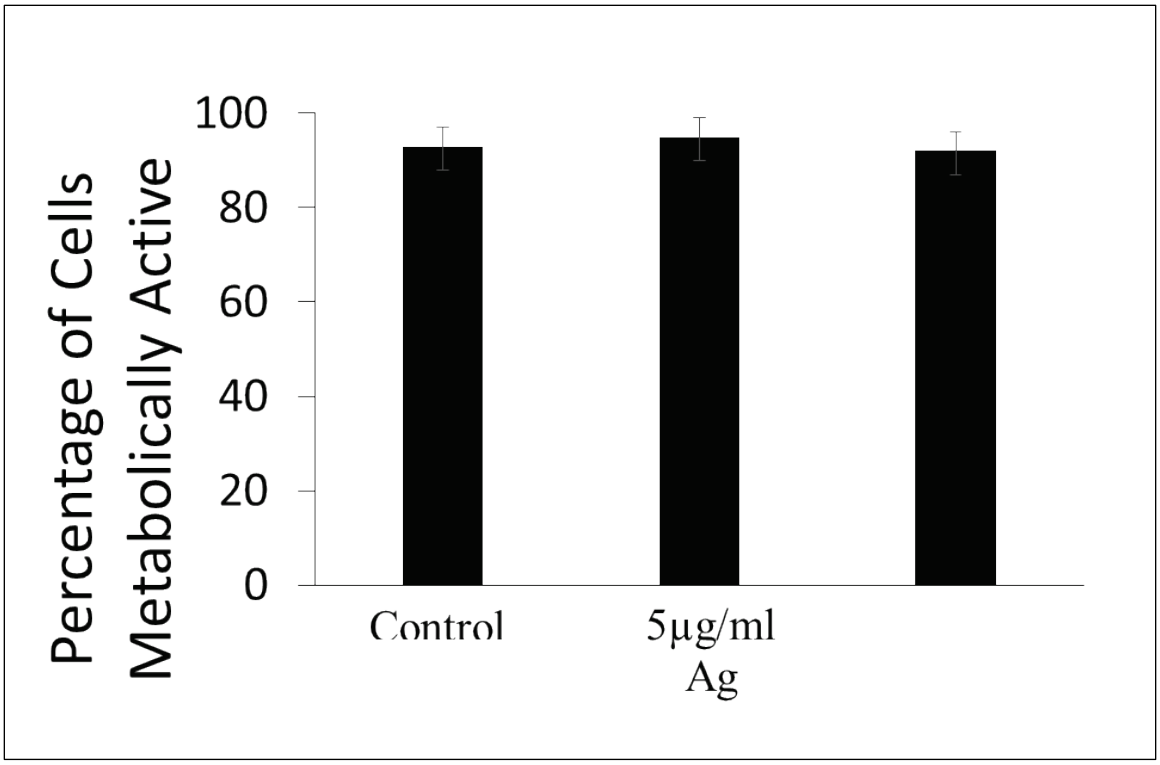
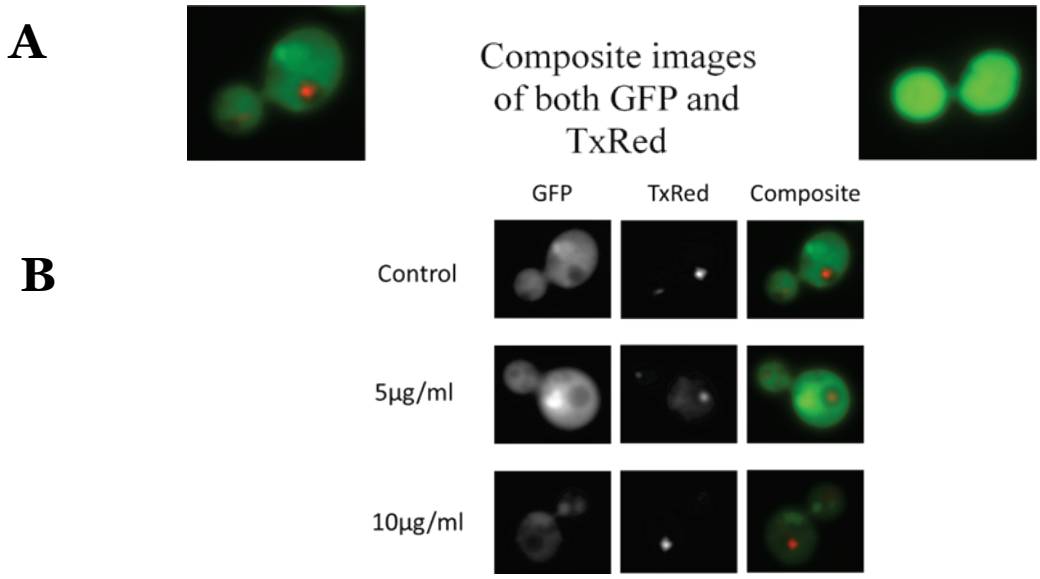


Figure 3. A. Example images of metabolically active (left), and inactive (right) cells. B. Representative images of cells treated with 5 µg/ml Ag ENMs , 10 µg/ml Ag ENMs, and control untreated cells stained with FUN-1 dye. Each condition yielded a similar count of cells with distinct red puncta in the vacuole, indicating metabolic activity.



5.3 Differential gene expression

Key results from the differential gene expression analysis are genes that are significantly differentially expressed in response to exposure. These genes can then be further investigated and organized by performing the following:

- Using the Saccharomyces Genome Database (SGD) (yeastgenome.org) go to menu>analyze>Gene Lists. Input your list of significantly differentially expressed genes. This database will then allow you to choose different categories of information you would like to receive about each gene. The protein it codes for, a description, and a phenotype description should be selected. The resulting table can be exported.
- Gene ontology terms of (1) molecular function, (2) subcellular localization and (3) biological process should be retrieved from the SGD. Menu>Analyze>GOSlimMapper should be used to produce a list of genes and their corresponding go terms, these results can then be plotted as histograms.

Figures 4–6 are examples of these results when testing Ag ENMs. Table 1 is a list of yeast genes significantly ($p \leq 5x10\text{-}5$) up or down regulated by fivefold change or higher in response to exposure to 5 µg/ml Ag ENM.

Figure 4. Histogram plots of G.O. Slim terms of each upregulated gene above a twofold change in 5µg/ml Ag ENM treatment. A total of 7126 genes were found, 483 genes were found to be upregulated above a twofold change in Ag ENM treated yeast.

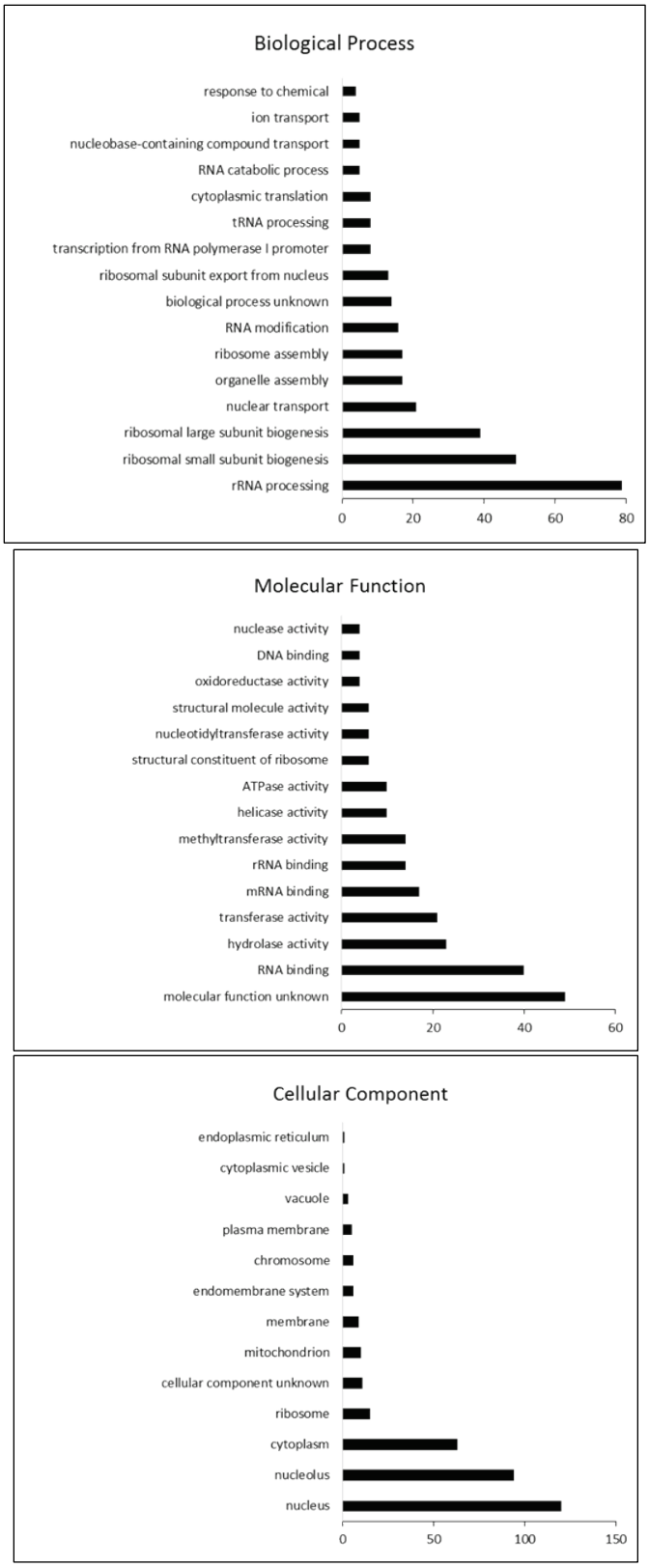


Figure 5. Histogram plots of G.O. Slim terms of each down regulated gene above a twofold change in 5µg/ml Ag ENM treatment. A total of 7126 genes were found, 92 genes were found to be downregulated by a twofold change or higher in Ag ENM treated yeast.

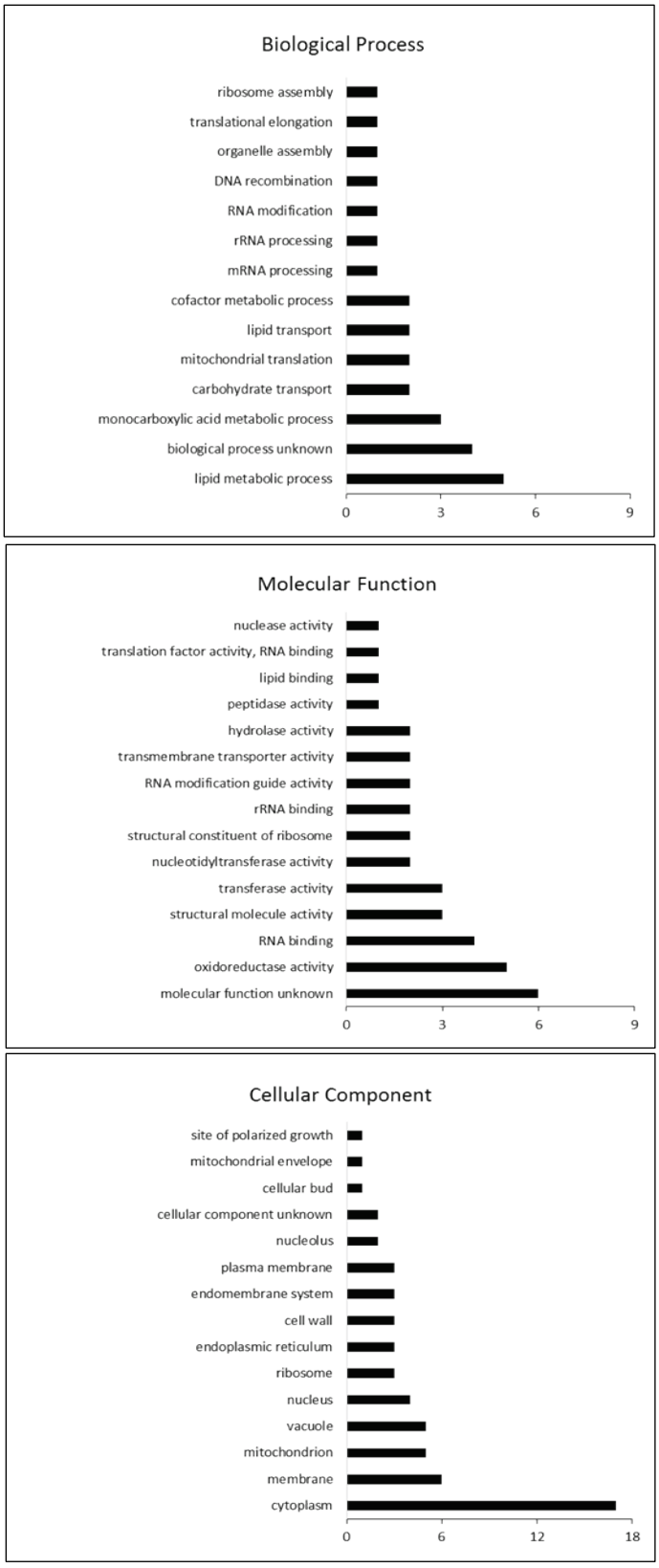


Figure 6. RT-qPCR result. More FAF1 and SDA1 transcripts are available in RNA samples obtained from silver nanoparti-cle-treated cell cultures. This results are consistent with the RNAseq results (See table 1). Therefore, it can be concluded that the RNAseq data is validated by RT-qPCR experiments.

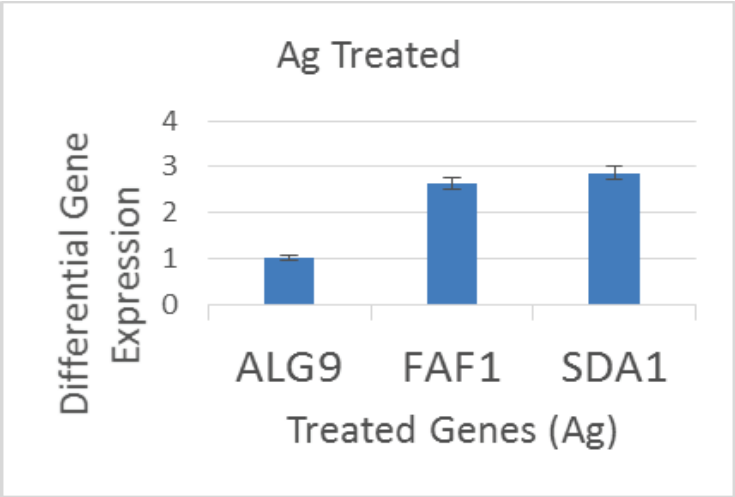


Table 1. List of Yeast genes significantly (p≤5x10-5) up or down regulated by fivefold change or higher in response to expo-sure to 5µg/ml Ag ENM.

Gene Name	Fold Change in Expression	Description
DAN1	-157.9	Cell wall mannoprotein
HES1	-7.5	Protein implicated in the regulation of ergosterol biosynthesis
ALD6	-7.0	Cytosolic aldehyde dehydrogenase
snR128	-6.8	C/D box small nucleolar RNA (snoRNA)
15S_rRNA	-6.8	Ribosomal RNA of the small mitochondrial ribosomal subunit
TIR1	-6.5	Cell wall mannoprotein
snR190	-5.3	C/D box small nucleolar RNA (snoRNA)
ERG5	-5.2	C-22 sterol desaturase
TMA23	5.0	Nucleolar protein implicated in ribosome biogenesis
RMT2	5.0	Arginine N5 methyltransferase
RRS1	5.0	Essential protein that binds ribosomal protein L11
RIX1	5.0	Component of the Rix1 complex and possibly pre-replicative complexes
DIM1	5.1	Essential 18S rRNA dimethylase (dimethyladenosine transferase)
RPA43	5.1	RNA polymerase I subunit A43
MTR4	5.2	ATP-dependent 3'-5' RNA helicase of the DExD/H family
MRD1	5.2	Essential conserved small ribosomal subunit (40s) synthesis factor
YTM1	5.3	Constituent of 66S pre-ribosomal particles

Gene Name	Fold Change in Expression	Description
CGR1	5.3	Protein involved in nucleolar integrity and processing of pre-rRNA
RBG1	5.4	Member of the DRG family of GTP-binding proteins
URA7	5.4	Major CTP synthase isozyme (see also URA8)
ROK1	5.4	RNA-dependent ATPase
TRM11	5.5	Catalytic subunit of adoMet-dependent tRNA methyltransferase complex
BUD23	5.5	Methyltransferase that methylates residue G1575 of 18S rRNA
NOP7	5.6	Component of several different pre-ribosomal particles
GFD2	5.7	Protein of unknown function
HCA4	5.7	DEAD box RNA helicase
YBL028C	5.8	Protein of unknown function that may interact with ribosomes
RRP36	6.0	Component of 90S preribosomes
DRS1	6.0	Nucleolar DEAD-box protein required for ribosome assembly and function
RRT14	6.0	Putative protein of unknown function
UTP23	6.1	Component of the small subunit processome
SYO1	6.3	Transport adaptor or symportin
NIP7	6.4	Nucleolar protein required for 60S ribosome subunit biogenesis
NMD3	6.8	Protein involved in nuclear export of the large ribosomal subunit
LTV1	7.0	Component of the GSE complex
RKI1	7.1	Ribose-5-phosphate ketol-isomerase
ENP2	7.4	Component of the SSU
RPF1	7.6	Protein involved in assembly and export of the large ribosomal subunit
CTR1	10.2	High-affinity copper transporter of plasma membrane
YDR433W	10.2	Dubious open reading frame
DBP2	10.2	ATP-dependent RNA helicase of the DEAD-box protein family
YPR123C	10.6	Dubious open reading frame
YML009W-B	10.6	Dubious open reading frame
FAF1	11.3	Protein required for pre-rRNA processing

6 Conclusion

This study found that at higher concentrations of silver ENMs, the ENMs precipitate out of media, causing high background noise in high concentration tests using the plate reader. Interaction between the nanoparticle and the media used for the cells should be determined and if possible, eliminated if present.

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Appendix A: Notes and Supplementary Data

A plate layout guide is shown in Table A-1.

Table A-1. General guide for 96-well plate set up.

ENM Comp(I):	100µL 2xTC(1)	100µL 2xTC(2)	100µL 2xTC(3)	100µL 2xTC(4)	100µL 2xTC(5)	100µL 2xTC(6)	100µL 2xTC(7)	...	100µL Buffer
CELL Comp(II)	100/µL Cell OD: 0.006 2xSD-Glu	100/µL Cell OD: 0.006 2xSD-Glu	100/µL Cell OD: 0.006 2xSD-Glu	100/µL Cell OD: 0.006 2xSD-Glu	100/µL Cell OD: 0.006 2xSD-Glu	100/µL Cell OD: 0.006 2xSD-Glu	100/µL Cell OD: 0.006 2xSD-Glu	...	100/µL Cell OD: 0.006 2xSD-Glu
BLANK Comp (III)	100µL 2xSD-Glu	100µL 2xSD-Glu	100µL 2xSD-Glu	100µL 2xSD-Glu	100µL 2xSD-Glu	100µL 2xSD-Glu	100µL 2xSD-Glu	...	100µL 2xSD-Glu
	1	2	3	4	5	6	7	...	12
A	I 1 &II	I 2 &II	I 3 &II	I 4 &II	I 5 &II	I 6 &II	I 7 &II	...	I 12 &II
B	I 1 &II	I 2 &II	I 3 &II	I 4 &II	I 5 &II	I 6 &II	I 7 &II	...	I 12 &II
C	I 1 &II	I 2 &II	I 3 &II	I 4 &II	I 5 &II	I 6 &II	I 7 &II	...	I 12 &II
D	I 1 &II	I 2 &II	I 3 &II	I 4 &II	I 5 &II	I 6 &II	I 7 &II	...	I 12 &II
E	I 1 &III	I 2 &III	I 3 &III	I 4 &III	I 5 &III	I 6 &III	I 7 &III	...	I 12 &III
F	I 1 &III	I 2 &III	I 3 &III	I 4 &III	I 5 &III	I 6 &III	I 7 &III	...	I 12 &III
G	I 1 &III	I 2 &III	I 3 &III	I 4 &III	I 5 &III	I 6 &III	I 7 &III	...	I 12 &III
H	I 1 &III	I 2 &III	I 3 &III	I 4 &III	I 5 &III	I 6 &III	I 7 &III	...	I 12 &III

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14. ABSTRACT

Engineered Nano Materials (ENMs) are commercially used in everyday products, including zinc sunscreens and water resistant fabrics and surfaces, but in the future, they may be used in targeted treatment of cancer, printable monitoring systems, and foldable phones. Understanding the effects of ENMs on the environment is crucial for the responsible use of these technologies. The aim of this project is to develop a standard operating procedure (SOP) for investigating the effects of ENMs on budding yeast (Saccharomyces cerevisiae). The ENMs used to develop this protocol were Ag and CdSe/ZnS. Toxicity was determined using plate assays to analyze the effect of ENMs on the growth cycle, Fun-1 staining assays to understand the effects on cell metabolism, and quantitative reverse transcriptase-based polymerase chain reaction (PCR) and RNAseq to understand the effects on genetic expression. From plate assays, doubling times, average time spent in lag phase, maximum concentrations were determined and compared between yeast grown in varying concentrations of ENMs, and a yeast grown in a control environment. Fun-1 staining determines the amount of metabolically active cells present in a treated cell culture. RNAseq and Quantitative PCR results, in expression levels of genes, are used to determine potential toxic effects of ENMs.

15. SUBJECT TERMS

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Saccharomyces cerevisiae

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