PQRI Workshop:
TiO2 Use in Pharmaceuticals
Global Regulatory and Technical Challenges
June 13-14, 2023

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

TDMA's New Science Program for TiO_2

David Lockley, Chair of TDMA Scientific Committee and
David Kirkland, Kirkland Consulting

PQRI Workshop, 13-14 June 2023 Bethesda, Maryland

A sector group of Cefic

TDMA's approach after the EFSA opinion

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

Bring forward trusted science

Engage with Authorities globally

Cooperate with TiO_2 stakeholders

Challenging scientific situation

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

A challenging landscape for the industry because:

1. TDMA provided all the requested data to EFSA for food additives showing no adverse outcomes

2. TiO_2 was the first in the line of a long list of similar substances

3. The assessment sets a precedent and there is no 'standard' response

Facts about TDMA's scientific commitment

EUR 13.55 million science programme launched and approved in 2018

Designed to fill any perceived gaps in safety data

Carry out studies to the latest guidelines and scientific techniques

Address emerging concerns

TDMA's approach to EFSA's opinion

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

efsa
European Food Safety Authority

EFSA 2021 E171 Opinion took a radical new approach to safety science

No pre-existing playbook to address the completely novel approach

TDMA sets up a TiO_2 Genotox Panel with independent experts to carry out scientific review and advise on response

In addition:

- **TDMA have held workshops in Slough and Cambridge with both experts and regulators that have shaped the science programme that TDMA are going to undertake over the next 3 years**

- **This will be managed by a Genotoxicity Working Group including experts on particle toxicity and Chaired by David Kirkland**

A sector group of Cefic

TDMA science programme:
Next steps

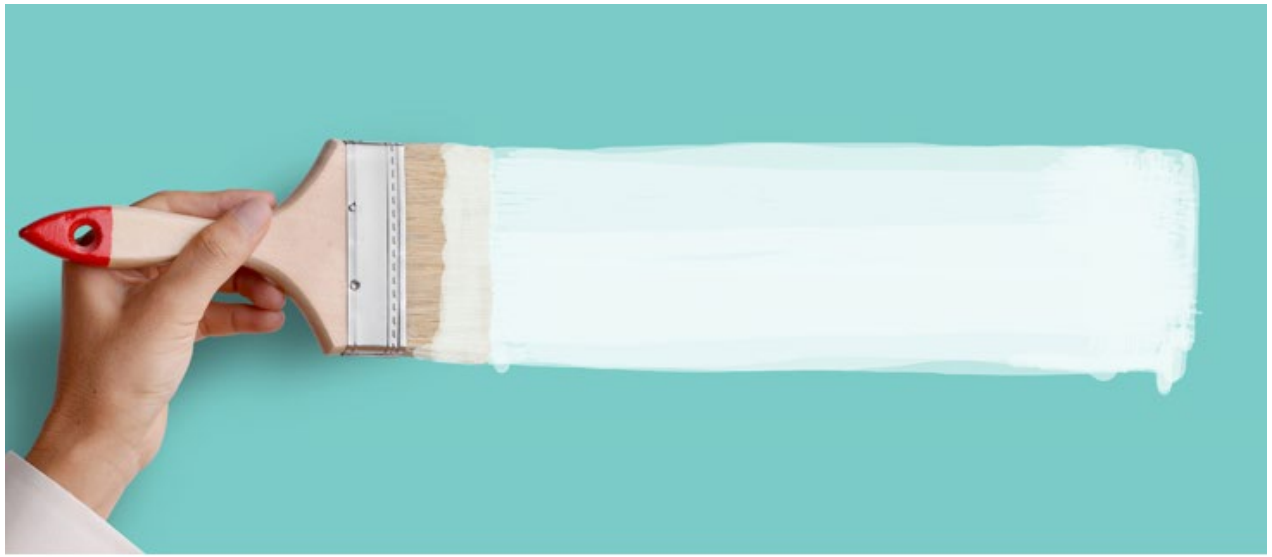
TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

- TDMA will keep engaging to have our work considered by all regulatory agencies including in the European Medicines Agency (EMA) and Joint FAO-WHO Expert Committee Report on Food Additives (JECFA) reviews
- TDMA experts submitted 200+ page dossier to JECFA in Feb 2023 and circulated around regulators globally
- TDMA is advocating that the European Commission triggers are review to relook at the science

Genotox panel published as paper	Investments in science based on recommendations	Ongoing scientific studies to deliver results
October 2022	**2023**	**2023 onwards**

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

Proposed strategy for “gap-filling” the genotoxicity profile of TiO_2



New studies

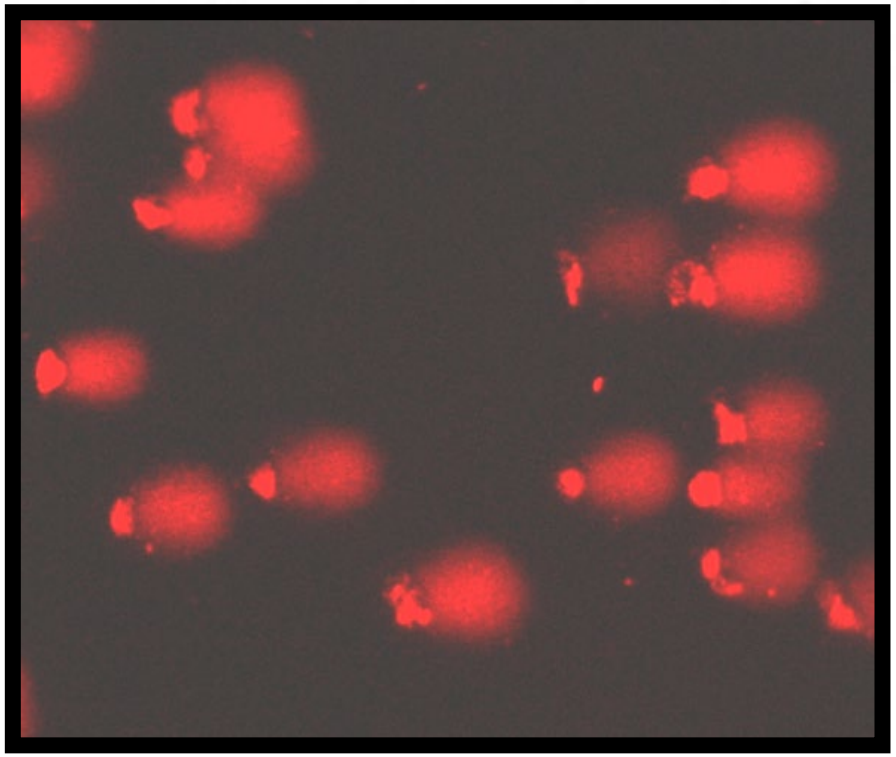
TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

- A study to measure induction of DNA strand breaks (comets) in lung following intratracheal instillation of 13 different grades of TiO_2 has been mandated for REACH substance evaluation
  - This is designed to select a smaller number of grades for full inhalation studies
- It is proposed to investigate induction of gene mutations in transgenic animals according to OECD guideline 488
- Further, research at University of Cambridge has shown that TiO_2 is sequestered in high amounts in macrophages (lysomac cells) of Peyer's patches (ileum area of the small intestine) following dietary administration to mice
  - Also seen in human samples
  - It is not known whether rats show the same effect, so this will be investigated

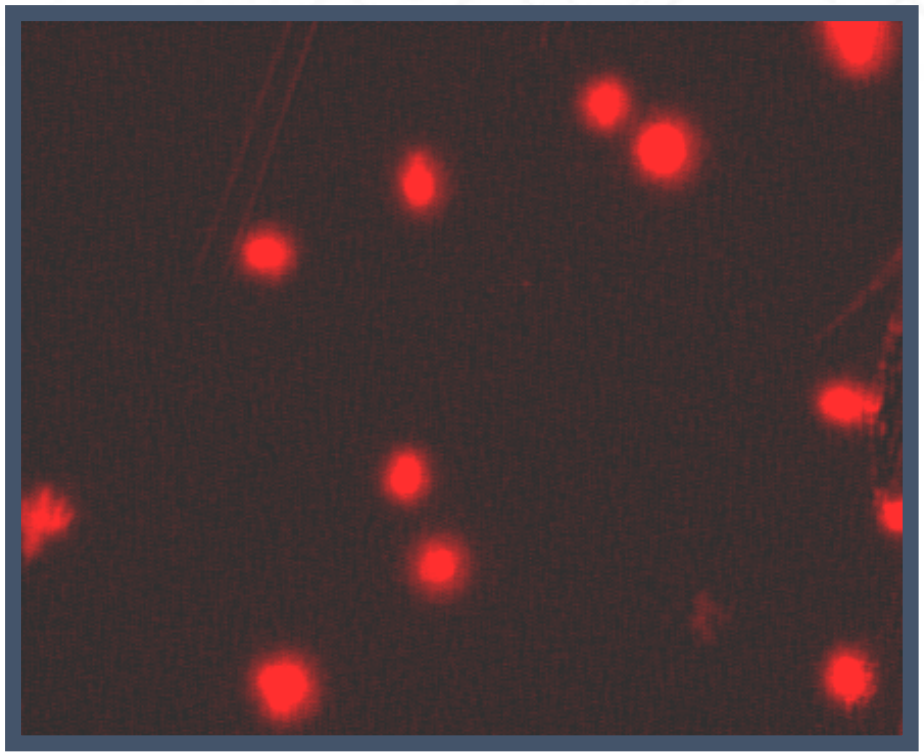
Representative photomicrographs of comets

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

Cells with DNA migration



Cells without DNA migration



REACH comet study

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

- This is a very challenging study because of:
  - The numbers of TiO_2 grades to be tested
  - Dosing on 2 consecutive days
  - Sampling lung tissue at 2-6 hrs, 24 hrs and 28 days after the 2nd dose
  - Additional investigations to look for oxidative stress, tissue toxicity, changes in blood parameters, cardiovascular function, histopathology etc.
- Because 3 concentrations of each grade of TiO_2 need to be included, together with negative (vehicle) controls, plus positive control (standard and for oxidative damage), it is impossible treat the required 5 rats/dose group at the same time.
  - An “inert” particle control should also be included
- Since dosing therefore has to be split across different days, it is important to control for day-to-day variability, and this is done by constructing a “block design”

Block design

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

Week 2 Mon	Tue	Wed	Thu	Fri
1st IT instillation **2 rats x TiO_2 Sample 1 (low dose)** **2 rats x TiO_2 Sample 1 (mid dose)** **2 rats x TiO_2 Sample 1 (high dose)** **2 rats x vehicle control**	2nd IT instillation	Sacrifice 24 h Technical positive controls (EMS, Pot. bromate)	Electrophoresis	
	1st IT instillation 3 x TiO_2 1 low 3 x TiO_2 1 mid 3 x TiO_2 1 high 3 x vehicle control	2nd IT instillation	Sacrifice 24 h Technical positive controls (EMS, Pot. bromate)	Electrophoresis

- Technical positive controls are samples of tissues or cells treated previously and “banked”. They allow the “comet” processing steps to be checked for acceptability.
- Groups of rats treated concurrently with positive control (EMS) will be included at certain intervals.
- An inert particulate negative control (BayerTitan T) will be tested as a separate test substance in the same manner.

Critical sampling

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

- The first sampling time for the comet assay (and most challenging in terms of resources) is 2-6 hours after the second instillation
  - We don't want to be sampling some groups 2 hrs and other groups 6 hrs after the 2nd instillation, because such variability could impact the results
- Also, stress caused by the instillation procedure could impact the results
- Proposal to dose 12 rats with vehicle and sample 2 rats each at 2, 3, 4, 5, 6 & 24 hrs, measure tail intensity (TI, comets) and inflammation (PMNs) and see when any "stress-related" effects have disappeared (i.e. TI and PMN levels fall to those seen at 24 hrs)
  - Can then "fix" that sampling time for all future early samples

General study objectives and endpoints

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

ECHA decision-based rat instillation study

This *in vivo* intratracheal instillation *s*tudy aimed at:

- **Gathering data on traditional BAL endpoints for 13 TiO_2 grades representing almost the whole market together with early and sensitive markers of toxicities as follows**:
  - **GLP**: BALF and histopathology of the lung, as required in the OECD Guidance Document n°39 (GD39) on inhalation studies (2018) -> mandatory LDH activity, total protein or albumin, total leukocyte count, absolute cell counts, and calculated differentials for alveolar macrophages, lymphocytes, neutrophils, and eosinophils.
  - **Non-GLP**: Oxidative stress in lung tissue measured by fluorimetric probes (e.g. DCFDA/H2DCFDA), malondialdehyde (MDA) and heme oxygenase-1 activity (not be performed at 28 days post-exposure).
  - **GLP**: Histopathology of liver, kidney, testis and brain
  - **Non-GLP**: Cardiovascular function by measure of endothelial nitrogen oxide synthase (eNOS) activity and high sensitivity C reactive protein (hs-CRP) content in the serum, if technically feasible.
  - **GLP:** hOGG1-modified (detection of oxidative DNA damage) alkaline comet assay (DNA strand break induction) with lung tissue (OECD 489)

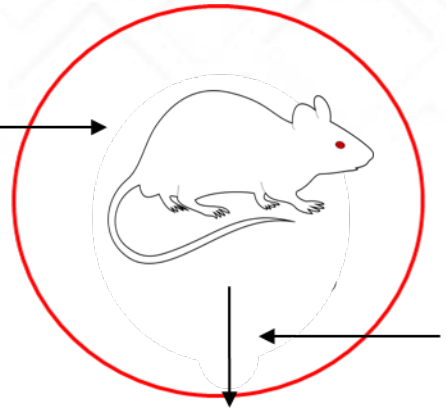
General study objectives and endpoints

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

ECHA decision-based rat instillation study

Alkaline Comet Assay : pH > 13

Intratracheal instillation of TiO_2 in rats



Preparation of single cells from lung tissue

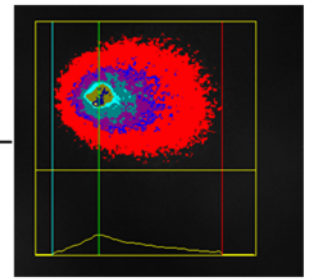
embedding of single cell suspensions into low melting agarose (LMA) on precoated slides

Incubation without or with hOGG1 enzyme

cell lysis over night or longer

pH > 13 → DNA-unwinding / electrophoresis

neutralization / DNA staining / analysis



© Fraunhofer ITEM

A sector group of Cefic

Preliminary work

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

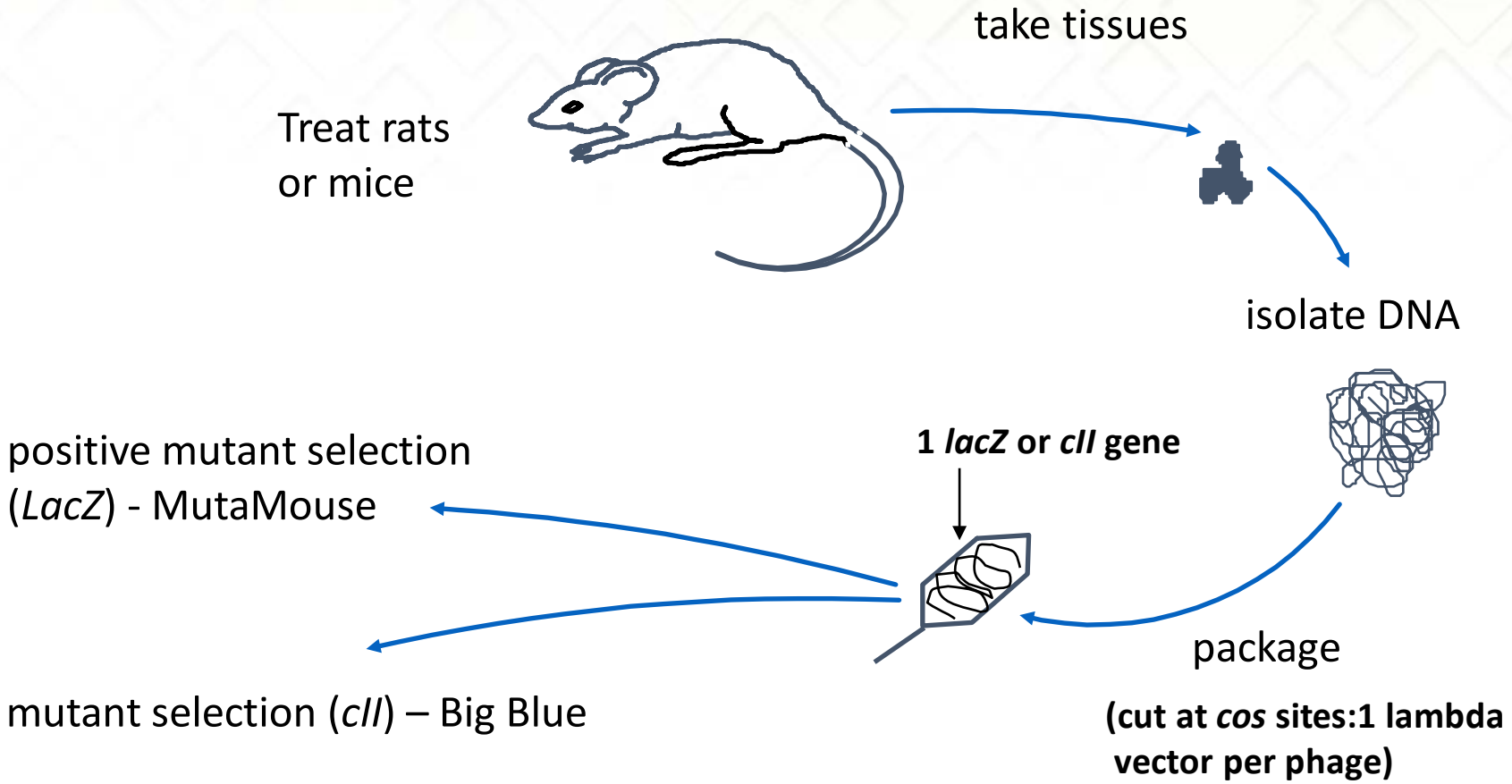
- Many of the “add on” investigations listed in the previous slide need to be evaluated in advance of the main study:
  - For reliability and reproducibility
  - To build historical control data that can be used to verify acceptability of methods during the main study and to aid interpretation of results
  - To check timing and resources needed
- The methods for providing “technical” positive controls also need to be established and checked
- A plan for the preliminary studies, and a draft protocol for the main study, will be reviewed by the sponsor and monitoring group
  - Site inspections may be needed for critical phases
  - Methods for detailed data recording need to be checked

Transgenic rodent gene mutation (TGR) study

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

- Rats or mice containing a viral vector into which a bacterial "reporter" gene (the transgene) is located are dosed daily for 28 days, and then, after allowing for mutations to be expressed, sampled on day 56
  - The choice of rats or mice will be determined after the preliminary Cambridge work (see later)
- Oral dosing is proposed, but some preliminary work will be done with E171 to determine whether this should be via diet, drinking water, or by gavage dosing
  - Need to investigate which route gives optimum exposure systemically (in blood) and in Peyer's patches
  - Need to identify maximum doses and set mid and low doses

Mutation in transgenes



Cohort	Animal#	Dosing method	Timing
1	Rat Fischer F344	TiO_2 in Diet*	28 day + 28 day recovery
2	Mouse C57BL/6	TiO_2 in Diet*	28 day + 28 day recovery
3	Rat Fischer F344	TiO_2 in Drinking water	28 day + 28 day recovery
4	Mouse C57BL/6	TiO_2 in Drinking water	28 day + 28 day recovery
5	Rat Fischer F344	TiO_2 by Gavage/sonication?	28 day + 28 day recovery
6	Mouse C57BL/6	TiO_2 by Gavage/sonication?	28 day + 28 day recovery
7	Mouse C57BL/6	TiO_2 in Diet*	16 weeks continuous feeding**

Wild-type strains of Big Blue transgenic rats and mice
*Exact feed formulation is defined by Cambridge
** To confirm previous findings with different strain of mice
Negative controls will be included.
In-life blood sampling for historical control data on day 29 for both 28 and 56 day cohorts.
Terminal blood, liver, spleen and Peyer's patches taken on day 29 and day 56.

Transgenic rodent gene mutation (TGR) study

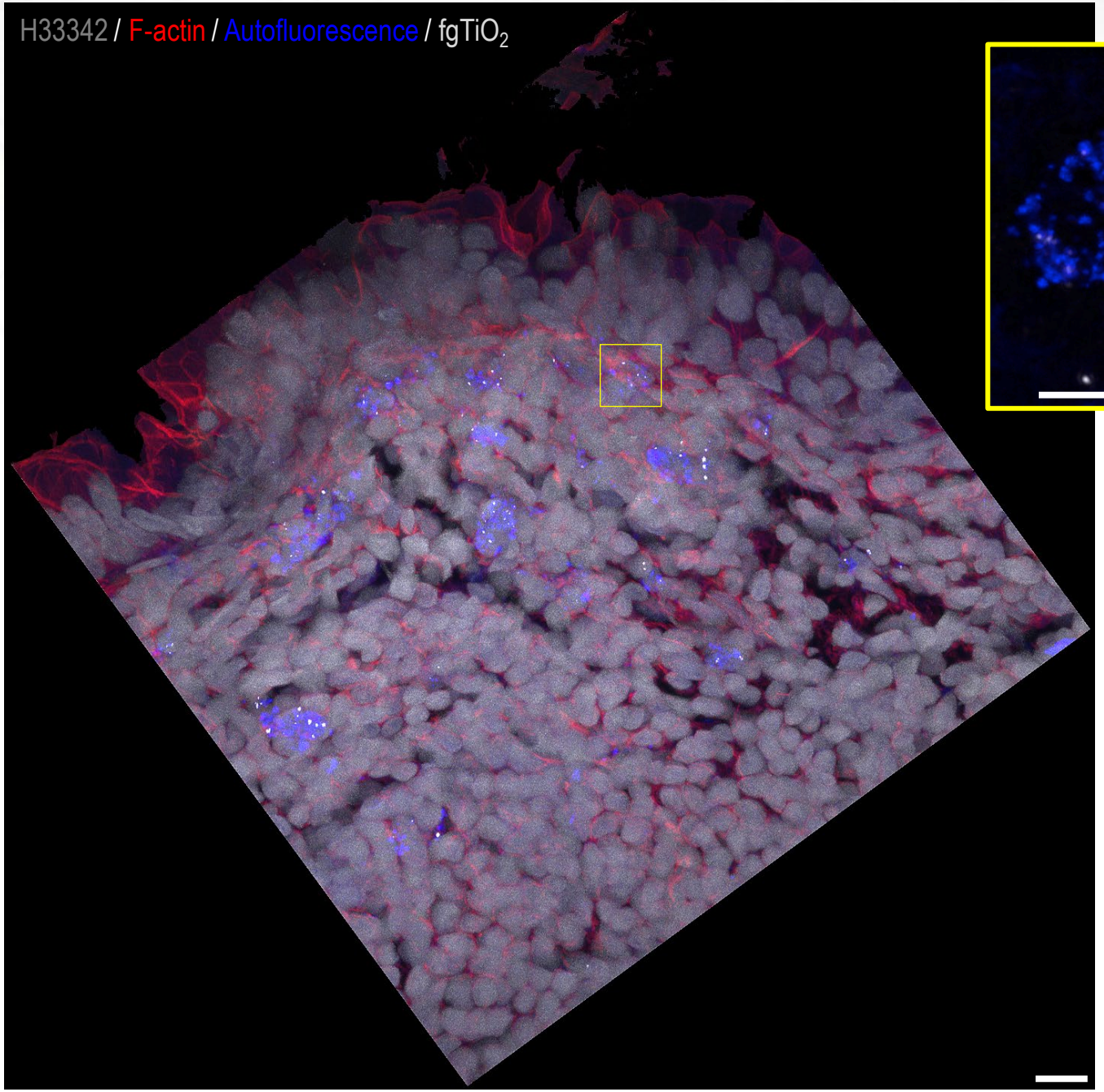
TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

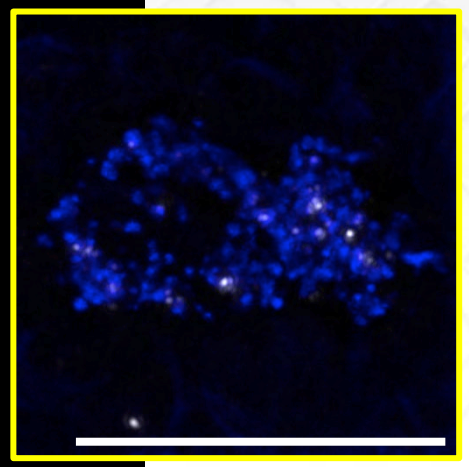
- The tissues sampled most routinely are glandular stomach and duodenum (site of contact effects) and liver (highest exposed internal organ and site of metabolism)
  - Bone marrow will also be sampled (peripheral, rapidly dividing tissue)
  - Ileum, colon, testes, spleen, kidney and brain will be sampled and frozen for possible later analysis
    - Some of these are being taken for histology in the instillation comet study
  - Blood samples will be taken at appropriate intervals for measurement of micronuclei in reticulocytes
  - Satellite animals will be treated and blood sampled for measurement of TiO_2 exposures
- The grades of TiO_2 to be tested in TGR main study will be determined from *in vitro* gene mutation tests on the 13 grades to be used in the lung comet study

Peyer's patches (John Wills studies) - 1

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

- Lysomac cells (differentiated macrophages) in Peyer's patches of mice (and humans) exposed by the oral route contain large amounts of TiO_2. The cells appear to persist for long periods.
- Whilst the lysomac cells do not divide, and therefore will not express mutations, or suffer any deleterious genotoxic effects, cells that surround them can divide and could experience genotoxic damage
  - So-called "by-stander effects"
- John Wills has frozen samples of Peyer's patches from treated and control mice which could be investigated for DNA damage using the γH2AX technique (detects double-stranded DNA breaks)





TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

Food grade TiO_2 selectively and specifically targets Peyer's patch 'lysomac' macrophages

A sector group of Cefic

Peyer's patches (John Wills studies) - 2

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

- If γH2AX lesions increase in "by-stander" cells of treated mice, then samples could be taken and analysed for presence of mutations
  - DNA strand breaks may be repaired or be lethal, and may not necessarily be converted to stable genetic changes such as mutations
- The amounts of tissue available surrounding TiO_2-rich lysomac cells will not provide sufficient DNA for the TGR technique to be used
- However, Duplex Sequencing (which is a version of a new technique called error-corrected next generation sequencing or ecNGS) requires much less tissue
  - Measures changes in DNA sequences, so pre-mutagenic lesions
- If the DNA strand breaks do not lead to pre-mutagenic lesions then it is highly likely they do not represent a direct genotoxic effect and may be secondary to other effects such as induction of oxidative stress
- Duplex Sequencing could also be done in the animals treated in the TGR study
  - Hence the rationale for freezing samples of ileum

Conclusion

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

- Growing consensus among key international regulatory authorities about the safety of E171
- TDMA is making progress in addressing the novel approach taken in the EFSA opinion
- TDMA will keep engaging with relevant stakeholders to address concerns and ensure relevant science on E171 safety is considered

TDMA
TITANIUM DIOXIDE MANUFACTURERS ASSOCIATION
for a brighter future

Thanks for your attention!

Contact: tdma@cefic.be

More information on tdma.info

A sector group of Cefic