United States Environmental Protection Agency

Development of an *In Vitro* Human Thyroid Microtissue Model

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The views expressed in this presentation are those of the presenter and do not necessarily reflect the views or policies of the U.S. EPA

US Environmental Protection Agency

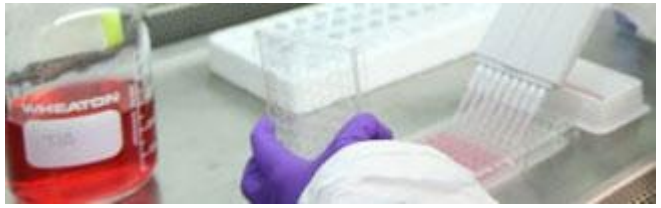
- **EPA Office of Research and Development**: Conducts leading-edge research to foster the sound use of science and technology to fulfill EPA's mission to protect human health and the environment.
- Research Triangle Park, NC location conducts science that has broad impacts on decision making at local, regional, and national levels.

US EPA Office of Research and Development
National Research Programs

Air & Energy

Homeland Security

Chemical Safety For Sustainability



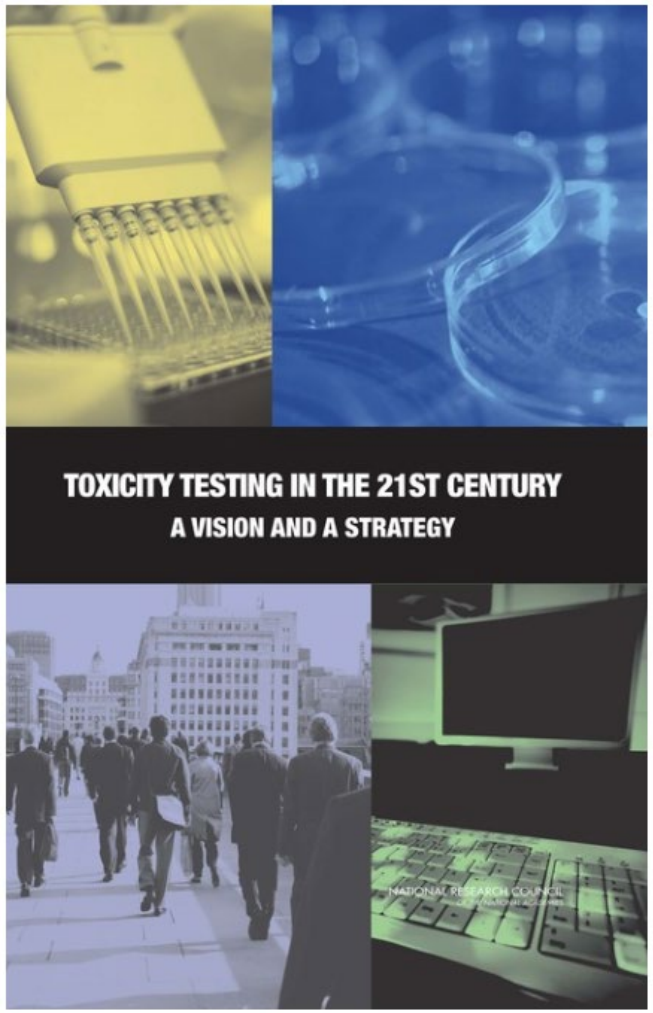
Health & Environmental Risk Assessment

Safe & Sustainable Water Resources

Sustainable & Healthy Communities

Deisenroth Lab
Research Program: Chemical Safety for Sustainability
Focus: Development and application of novel assay technologies for chemical hazard identification and prioritization.

Toxicity Testing in the 21st Century



National Research Council released a report in 2007 calling for a genuine commitment to the **reduction, refinement, and replacement** of animal testing:

- Increase data relevance with human cell-based testing
- Speed up the data gathering process by transitioning away from animal models to high-throughput screening
- Focus on mechanisms of toxicity by integrating molecular biology with toxicology
- Incorporate elements of population and exposure into every risk assessment
- Suggestions grounded in the political and economic landscape of global chemical testing

http://nap.edu/11970. DOI 10.17226/11970

The Frank R. Lautenberg Chemical Safety for the 21st Century Act

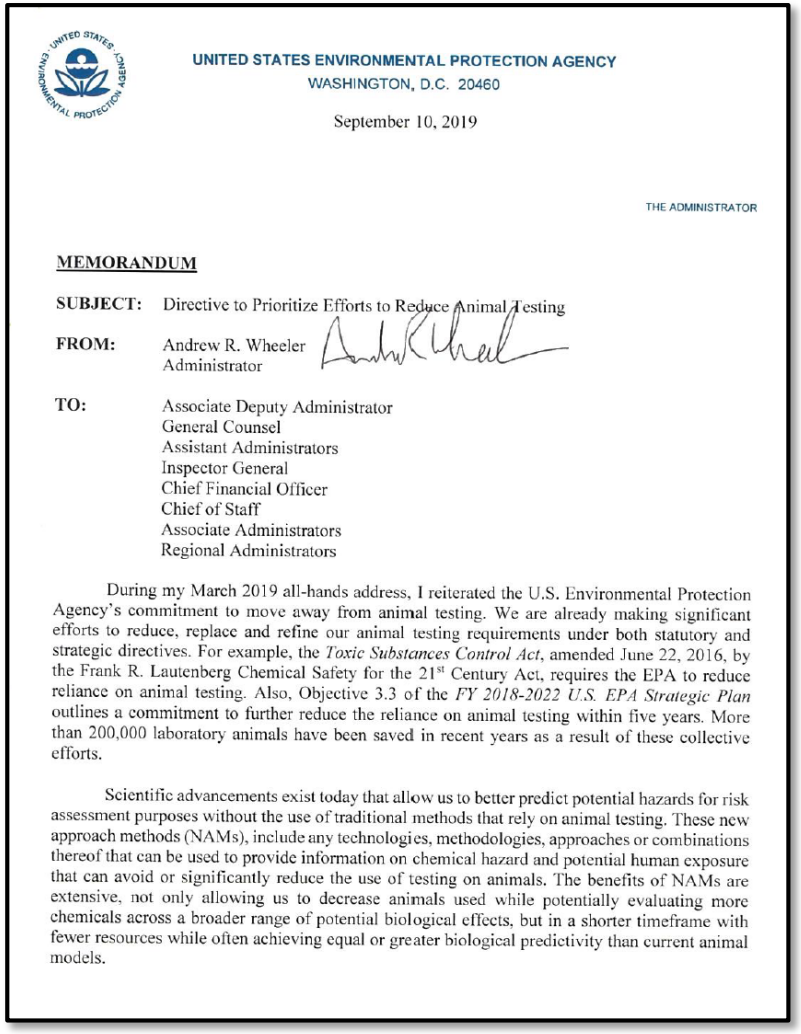
- Amendment of the Toxic Substances Control Act (TSCA), the nation's primary chemical management law

- Mandatory requirement for EPA to evaluate existing chemicals, establish risk-based safety standards, increase public transparency for chemical information, provide consistent source of funding for EPA to carry out responsibilities in the law.

- Section 4(h) in the new TSCA legislation requires –
  - "…Administrator shall reduce and replace, to the extent practicable and scientifically justified…the use of vertebrate animals in the testing of chemical substances or mixtures…"
  - Alternative approaches need to provide "information of equivalent or better scientific quality and relevance…" than the traditional animal models

https://www.congress.gov/114/plaws/publ182/PLAW-114publ182.pdf

Agency Goals for Reduction in Animal Testing



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

September 10, 2019

THE ADMINISTRATOR

MEMORANDUM

SUBJECT: Directive to Prioritize Efforts to Reduce Animal Testing

FROM: Andrew R. Wheeler
Administrator

TO: Associate Deputy Administrator
General Counsel
Assistant Administrators
Inspector General
Chief Financial Officer
Chief of Staff
Associate Administrators
Regional Administrators

During my March 2019 all-hands address, I reiterated the U.S. Environmental Protection Agency's commitment to move away from animal testing. We are already making significant efforts to reduce, replace and refine our animal testing requirements under both statutory and strategic directives. For example, the *Toxic Substances Control Act*, amended June 22, 2016, by the Frank R. Lautenberg Chemical Safety for the 21st Century Act, requires the EPA to reduce reliance on animal testing. Also, Objective 3.3 of the *FY 2018-2022 U.S. EPA Strategic Plan* outlines a commitment to further reduce the reliance on animal testing within five years. More than 200,000 laboratory animals have been saved in recent years as a result of these collective efforts.

Scientific advancements exist today that allow us to better predict potential hazards for risk assessment purposes without the use of traditional methods that rely on animal testing. These new approach methods (NAMs), include any technologies, methodologies, approaches or combinations thereof that can be used to provide information on chemical hazard and potential human exposure that can avoid or significantly reduce the use of testing on animals. The benefits of NAMs are extensive, not only allowing us to decrease animals used while potentially evaluating more chemicals across a broader range of potential biological effects, but in a shorter timeframe with fewer resources while often achieving equal or greater biological predictivity than current animal models.

Goals

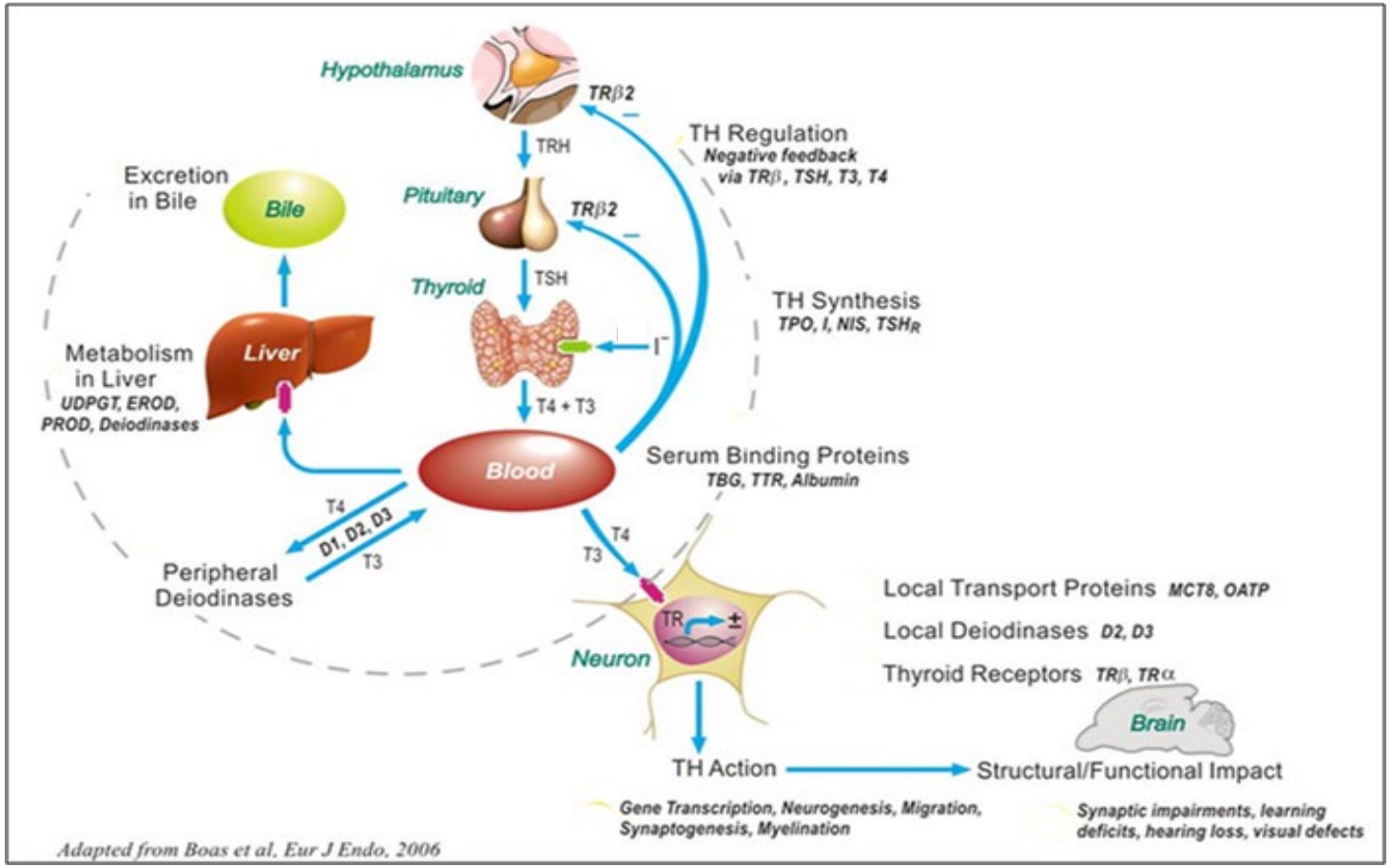
- Reduce requests for, and funding of, mammalian studies by 30% by 2025
- Eliminate all mammalian study requests and funding by 2035
- Come as close as possible to excluding reliance on mammalian studies from its approval process (subject to applicable legal requirements).
- *Exceptions may be granted on a case-by-case basis*

Objectives

- Evaluate regulatory flexibility for accommodating the use of new approach methods (NAMs)
- Develop baselines and metrics for assessing progress
- Validation to ensure NAMs are equivalent to or better than the animal tests
- Demonstration that NAMs are applicable for use in risk assessment and protective of human health and environment
- Engage and communicate with stakeholders

https://www.epa.gov/sites/production/files/2019-09/documents/image2019-09-09-231249.pdf

Endocrine Toxicology: Why Do We Care About Thyroid?



- Thyroid hormones are essential for normal growth, development, cell differentiation, and energy homeostasis.
- Thyroid dysfunction is characterized by under- (hypothyroidism) or over- (hyperthyroidism) activity of the gland.
- Thyroid dysfunction has an impact on four major adverse health outcomes:
  - Neurodevelopment and function
  - Cancer
  - Cardiovascular disease
  - Lipid metabolism
- Environmental chemical exposures associated with thyroid dysfunction:
  - Perchlorate and thiocyanate (with iodine deficiency)
  - Mercury and arsenic
  - Certain organochlorine pesticides, poly-aromatic hydrocarbons, and perfluorinated compounds

European Commission. 2018. DOI: 10.2779/21828

Endocrine Disruptor Screening Program

Endocrine Pathway	Tier 1 Screening Battery											Tier 2 Testing Assays			
	ER Binding	*ERα Transcriptional Activation**	*AR Binding*	*Aromatase Inhibition*	*Steroidogenesis**	Uterotrophic*	Hershberger*	Pubertal Male	Pubertal Female	Amphibian Metamorphosis*	Fish Short Term Reproduction*	Rat 2-gen/ Extended One-Gen*	Medaka Extended One-Gen Repro Test*	Amphibian Growth and Dev Assay*	Japanese Quail Two Gen Toxicity Test
E+	■	■			■	■			■		■	■	■	■	■
E-	■			■	■				■		■	■	■	■	■
A+			■		■		■	■			■	■	■	■	■
A-			■		■		■	■			■	■	■	■	■
HPT Axis								■	■	■		■		■	■

The current EDSP assay battery evaluates effects of chemical exposures on estrogen, androgen, and thyroid endocrine pathways

- No *in vitro* tests for thyroid endpoints
- No human representation for thyroid
- Too reliant on animal tests

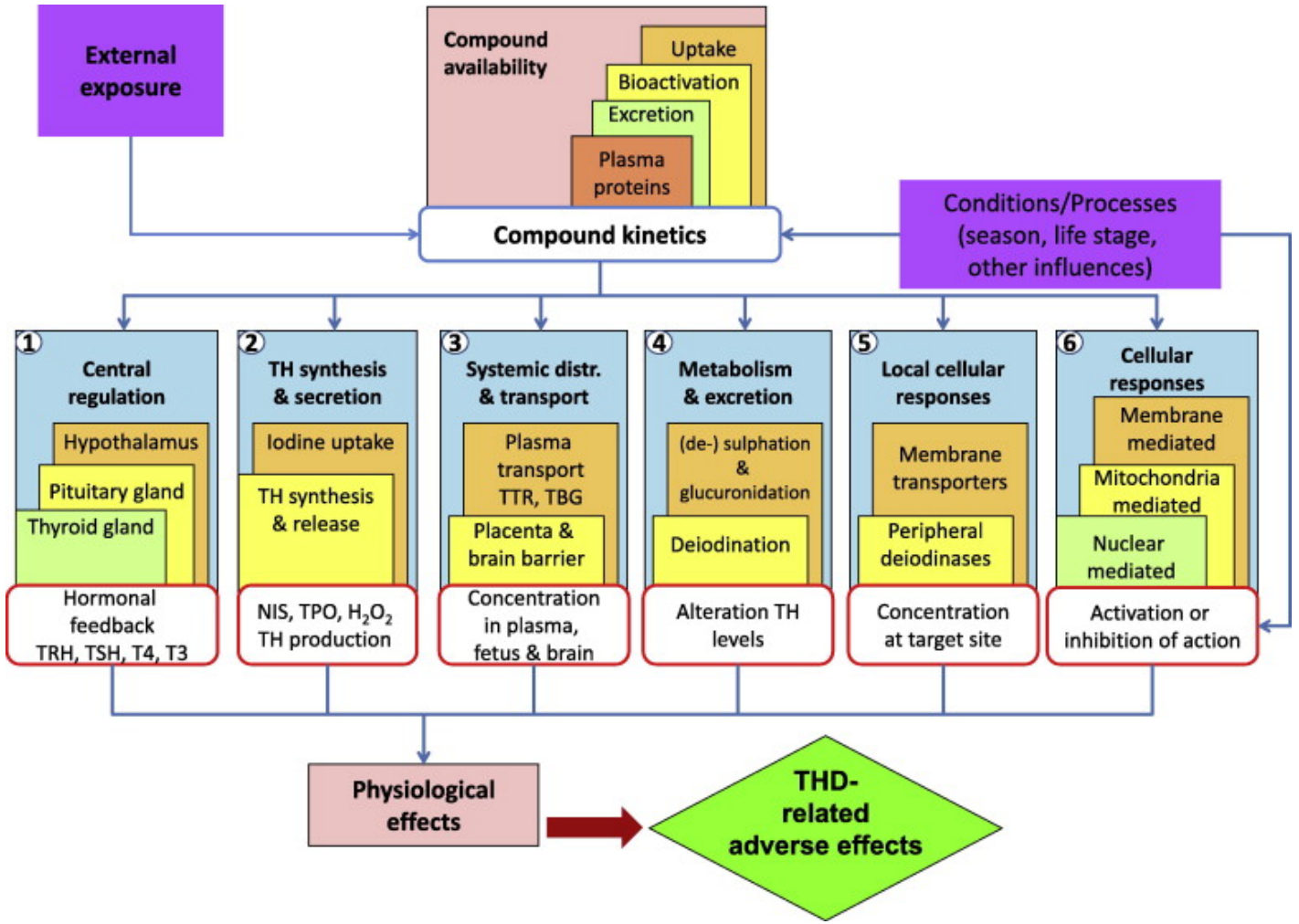
***In vivo* endpoints for thyroid-related endocrine testing in guideline studies**

- Serum T4 and TSH
- Thyroid and Pituitary weights
- Thyroid Histopathology

Screening Assay	Thyroid weight	Pituitary weight	Thyroid Histopathology	Serum TH levels
OECD TG 407	+	+	+	+ (optional)
OECD TG 408	-	-	+	-
OECD TG 416	+	+	-	-
OECD TG 422	-	-	+	-
OECD TG 441	-	-	-	+ (T3 and T4, optional)
OECD TG 443	+	+	+ (optional)	+ (T4 and TSH)
OECD TG 451			+	
OECD TG 452	+		+	
OECD TG 453	+		+	
EPA 15-day intact adult male rat assay	+	-	+	+
EPA Pubertal male	+	+	+	+ (T4 and TSH)
EPA Pubertal female	+	+	+	+ (T4 and TSH)

Jomaa, B. (2015).

Mapping Mechanism-based Testing Strategies for Thyroid Hormone Homeostasis

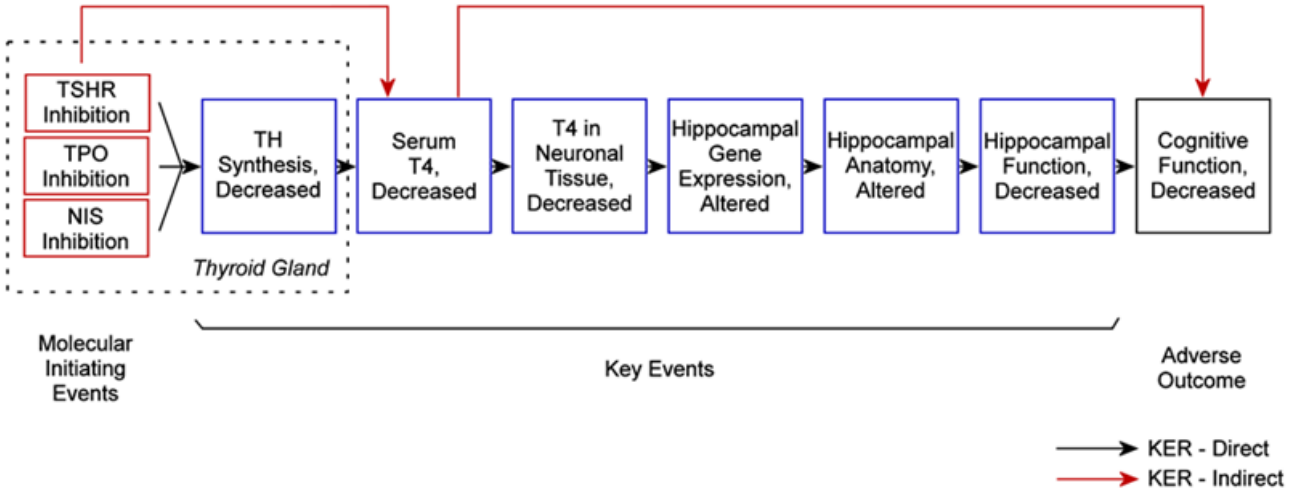


Murk, A. J. *et al.* Mechanism-based testing strategy using in vitro approaches for identification of thyroid hormone disrupting chemicals. *Toxicology in vitro.* (2013).

OECD New Scoping Document on in vitro and ex vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling. (2017).

EPA Continuing development of alternative high-throughput screens to determine endocrine disruption, focusing on androgen receptor, steroidogenesis, and thyroid pathways. *FIFRA SAP, November 28-30.* (2017).

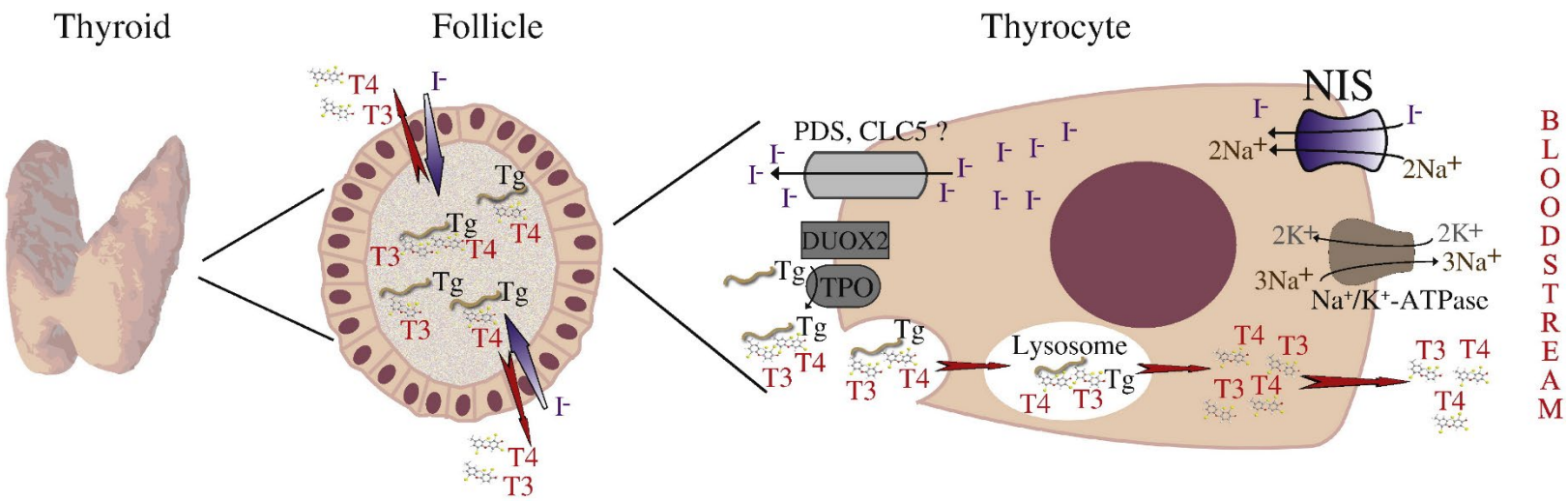
Challenges with *In Vitro* Thyroid Testing: Predicting Thyroid Hormone Disruption from High-throughput Assays



- The uncertainty surrounding the specificity of active chemicals identified in thyroid gland-related screens and the relevance to phenotypic effects on *in vivo* human thyroid hormone synthesis are notable data gaps for hazard identification of thyroid disrupting chemicals (TDCs).
- Additional data supporting indirect key event relationships (KER) could support predictive modeling of TDCs.

Target Gene	Assay	Environmental Chemicals Screened	Active Chemicals	% Active	Reference
TSHR	Engineered Cell Line	7871	825	10	TCPL: TOX21_TSHR_Agonist, TOX21_TSHR_Antagonist
TPO	Microsomal Enzyme	1074	314	29	K. Paul Friedman et al, ToxSci, 151(1), 2016, 160-180
NIS	Engineered Cell Line	293	137	47	J. Wang et al, EnvironSciTechn, 52, 2018, 5417-5426
NIS	Engineered Cell Line	768	172	22	J. Wang et al, Environment International, 126, 2019, 377-386
DIO 1	Recombinant Enzyme	292	50	17	M. Horning et al, ToxSci, 162(2), 2018, 570–581
DIO 1	Recombinant Enzyme	1819	221	12	J. Olker et al, ToxSci, 168(2), 2019, 430-442
DIO 2	Recombinant Enzyme	1819	303	17	J. Olker et al, ToxSci, 168(2), 2019, 430-442
IYD	Recombinant Enzyme	293	28	10	J. Olker et al, 2019, 58th SOT Annual Meeting

Challenges with *In Vitro* Thyroid Testing: Cell Type and Architecture are Critical Determinants for Hormone Synthesis



Cell Type

- No primary or thyroid cell lines, of any species, demonstrate appreciable capacity for thyroid hormone synthesis in 2D models
- Primary thyrocytes lose essential functions when cultured in conventional monolayer systems

Cell Architecture

- Follicular morphology is a critical feature for retaining hormone synthesis dynamics

Biochim Biophys Acta. 2014 Jan;1838(1 Pt B):244-53

Fit-for-Purpose Assay Design

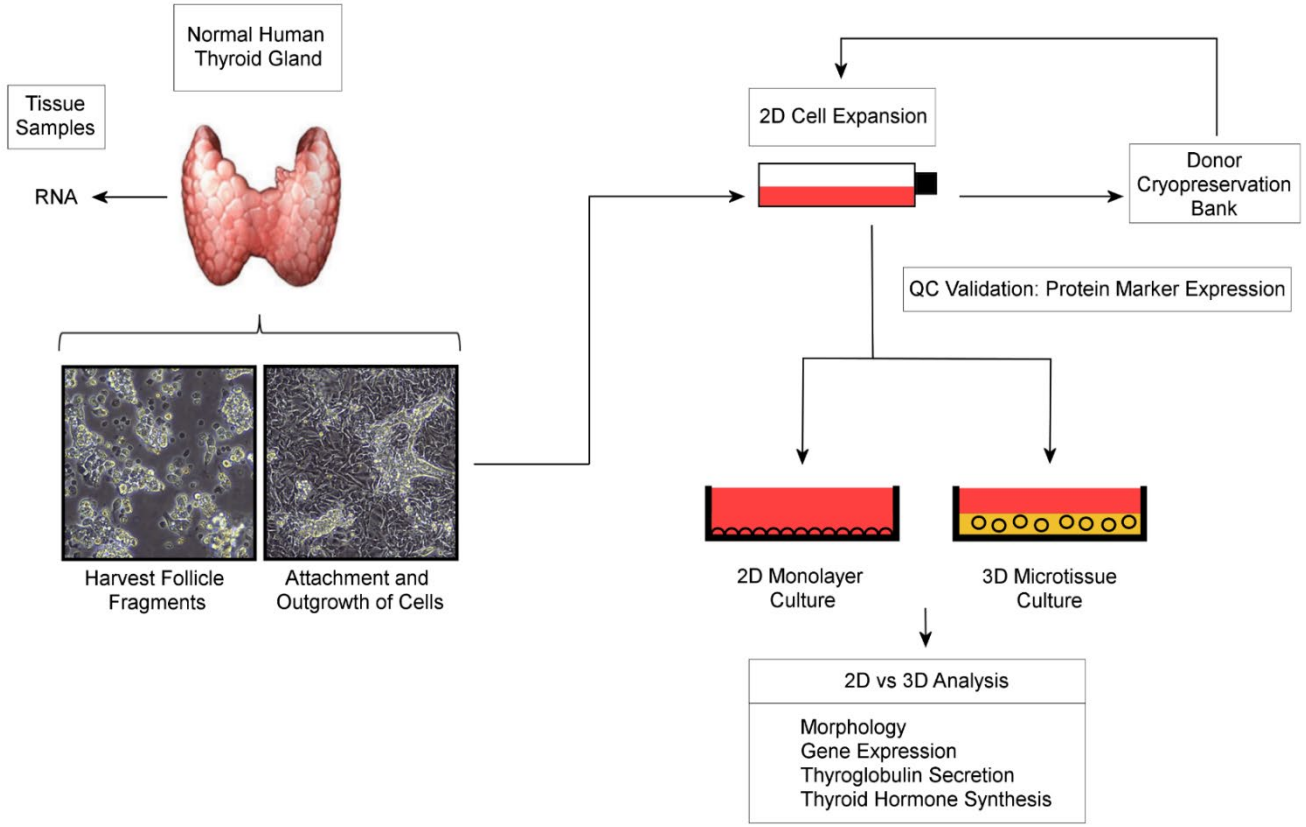
OXFORD
SOT | Society of Toxicology
academic.oup.com/toxsci

TOXICOLOGICAL SCIENCES, 174(1), 2020, 63–78

doi: 10.1093/toxsci/kfz238
Advance Access Publication Date: December 6, 2019
Research Article

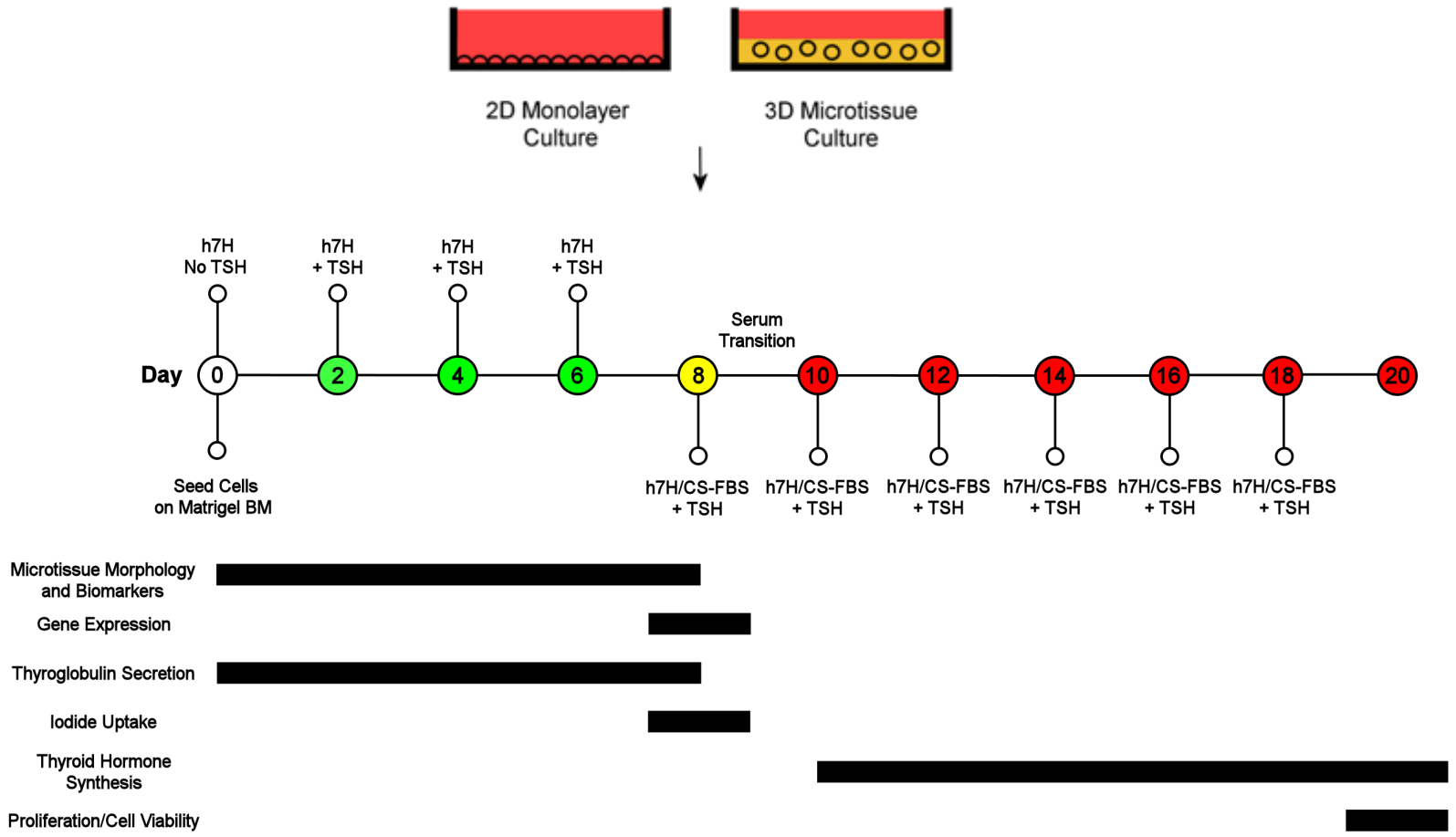
Development of an *In Vitro* Human Thyroid Microtissue Model for Chemical Screening

Chad Deisenroth ,*,[1] Valerie Y. Soldatow,[†] Jermaine Ford,[‡] Wendy Stewart,* Cassandra Brinkman,* Edward L. LeCluyse,[†] Denise K. MacMillan,[‡] and Russell S. Thomas *



Study objective: Develop a medium-throughput organotypic screening assay comprised of reconstructed human thyroid microtissues to quantitatively evaluate the disruptive effects of chemicals on thyroid hormone synthesis and secretion.

Experimental Design: Characterization of 2D vs 3D Culture Formats



Culture Model Design Specifications	
Donors	Multiple (Tissue and Cells)
Cells	Human Primary Thyrocytes
Culture Format	2D vs 3D
Extracellular Matrix	2D (None)
	3D (Matrigel)
Plate Format	96 well
Culture Medium	Humanized 7 homeostatic additives (h7H) medium
Serum	FBS (Day 0-8)
	CS-FBS (Day 8-20)
TSH Exposures	0, 1, 5 mU/ml
Incubation Period	0-20 Days

Thyroid Procurement: LifeNet Health Institute of Regenerative Medicine

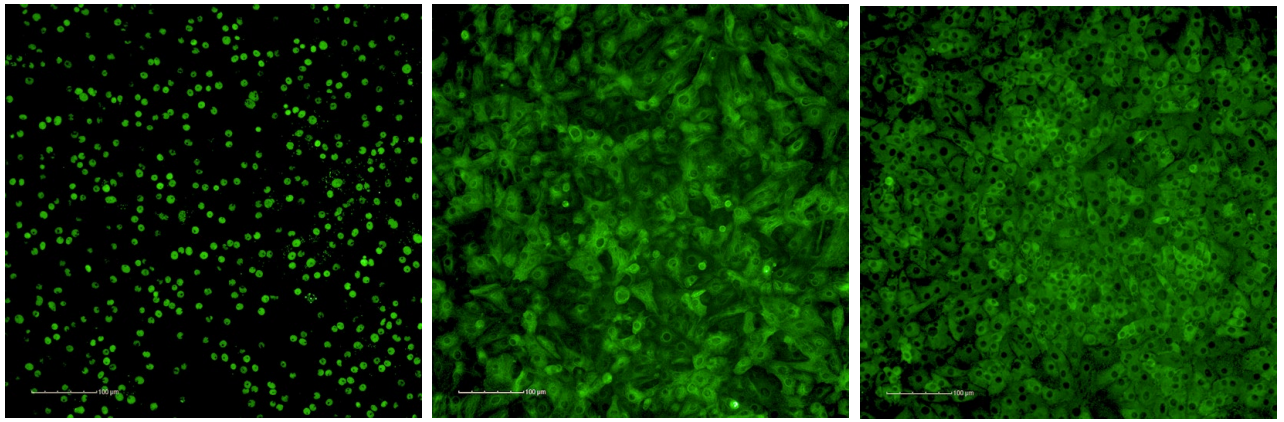
- Institute of Regenerative Medicine develops innovative or novel uses of donor tissues and organs through sound scientific and clinical research
- **Thyroid:** Procurement, digestion, expansion, cryopreservation

Donor	LNH ID	Age	Gender	Race	BMI
1	1721880	32	M	Caucasian	22
2	1722161	21	M	Caucasian	32
3	1811621	66	M	African American	35
4	1817005	27	M	Caucasian	19
5	1818646	31	M	Caucasian	31
6	1910289	18	M	Caucasian	22
7	1910552	36	M	Caucasian	37
8	1910594	17	M	African American	27

Table 1. Donor Specifications. LifeNet Health donor identification number (LNH ID) for all eight euthyroid donors examined in this study. Specifications for age, gender, race, and body mass index (BMI) are noted.

Mean Age: 31 [Range:17-66] years
Gender: Male
Race: Caucasian and African American
Mean Body Mass Index: 28 [Range: 19-37]

Donor Thyrocyte Characterization: Enrichment of Follicular Epithelial Cells

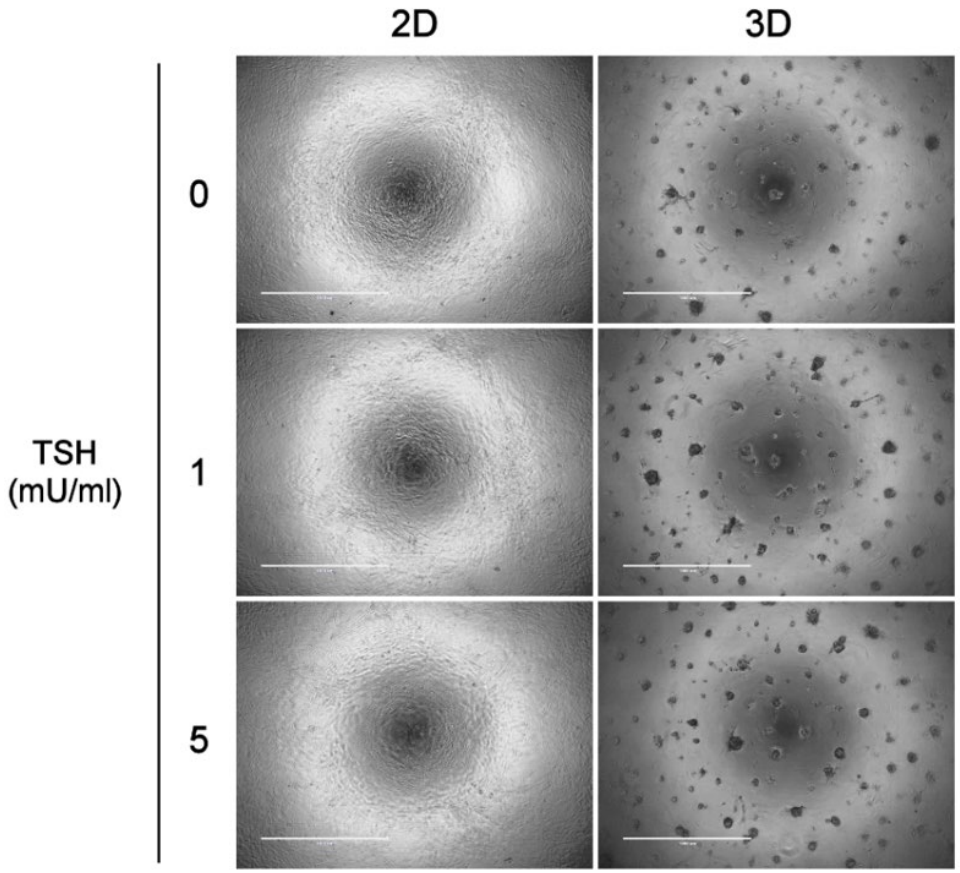


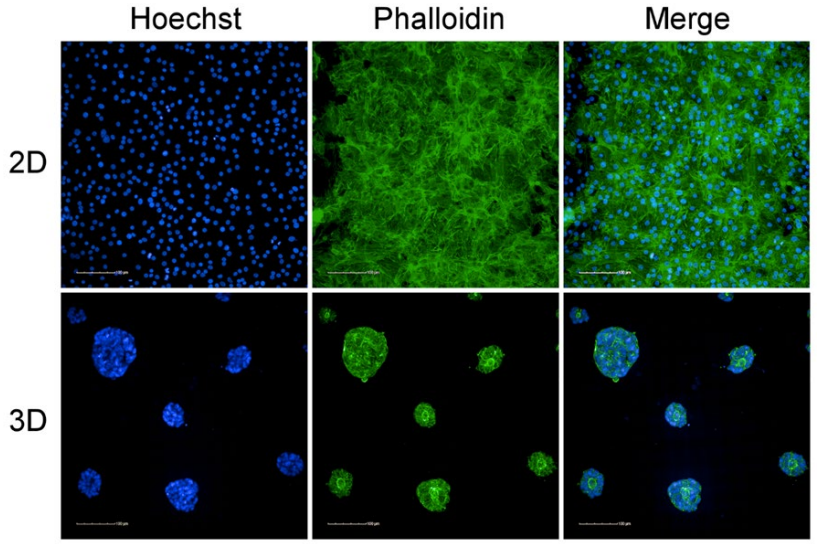
	IgG			IgG, kappa			NKX2-1			KRT7			TG		
Biomarker	**% POS**	**SEM**	**N**	**% POS**	**SEM**	**N**	**% POS**	**SEM**	**N**	**% POS**	**SEM**	**N**	**% POS**	**SEM**	**N**
NKX2-1	1.91	0.50	6	-	-	-	95.18	1.74	6	-	-	-	-	-	-
KRT7	-	-	-	0.30	0.14	6	-	-	-	90.52	2.47	6	-	-	-
TG	-	-	-	1.93	1.31	6	-	-	-	-	-	-	53.37	16.10	6

Donors LNH 1722161, 1817005, 1818646, 1910289, 1910552, 1910594

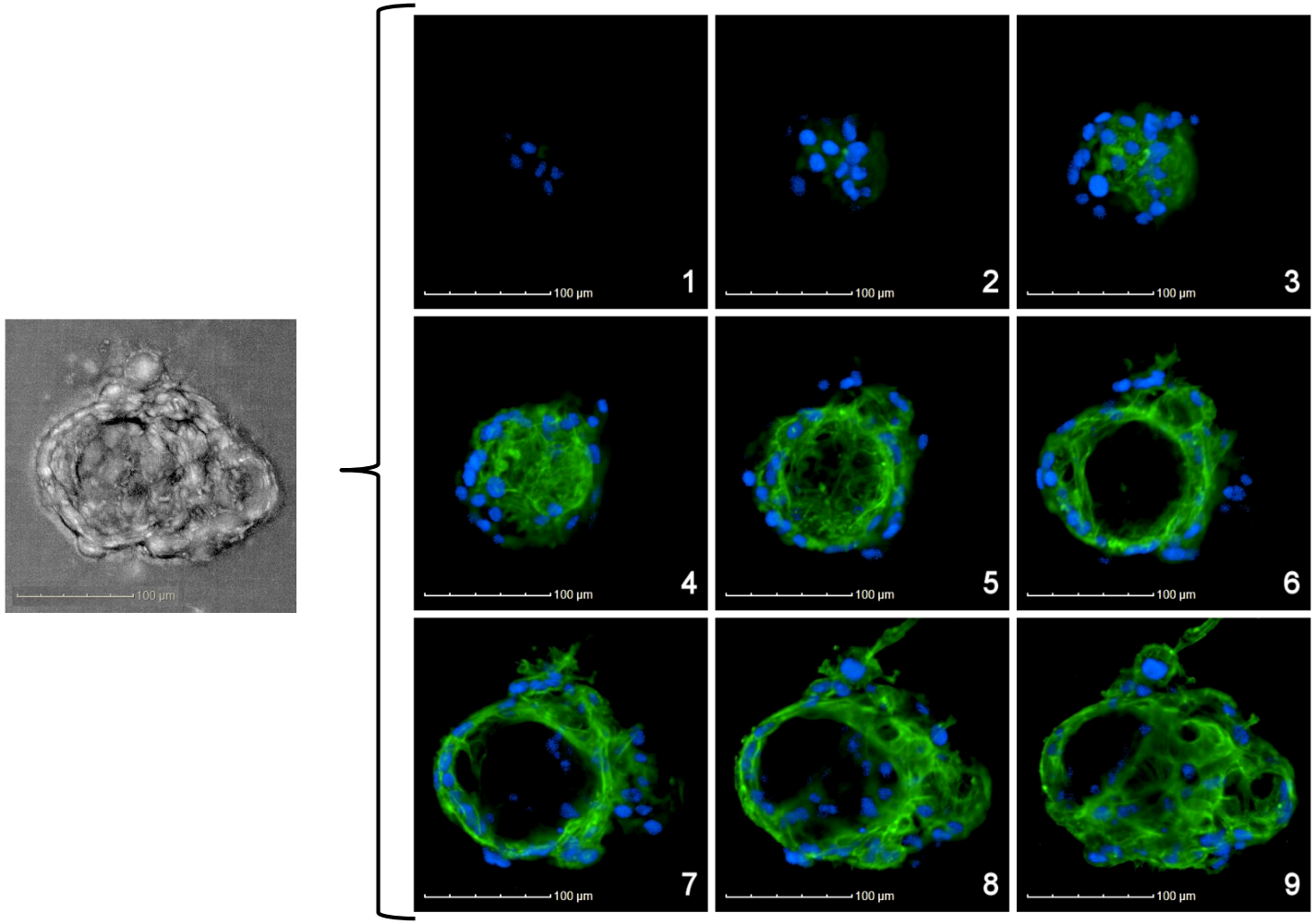
Table 3. Biomarker Image Cytometry. The cell-level frequency of IgG isotype controls (α-Mouse IgG kappa and α-Rat IgG), NK2 Homeobox 1 (NKX2-1), Keratin 7 (KRT7), and Thyroglobulin (TG) staining were quantitatively evaluated by high-content imaging across 6 independent human donors for verification of thyroid follicular epithelial cell enrichment. Data are the summary statistics presented as mean % positive (% Pos) ± SEM (n=6).

Donor Thyrocyte Characterization: 2D vs 3D Morphology





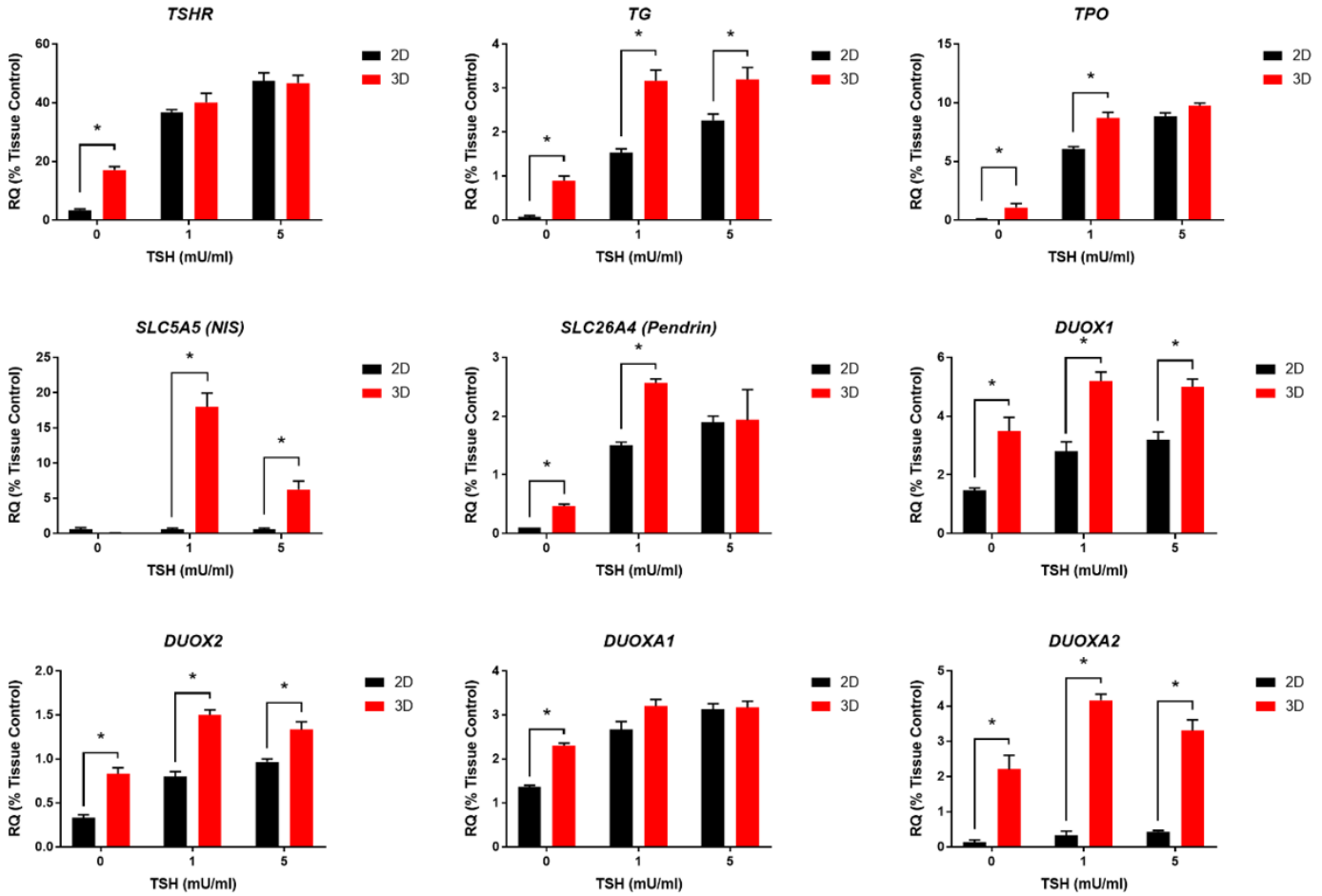
Donor Thyrocyte Characterization: Follicle-like Architecture



Donor LNH1722161: Confocal series of 3D microtissue.

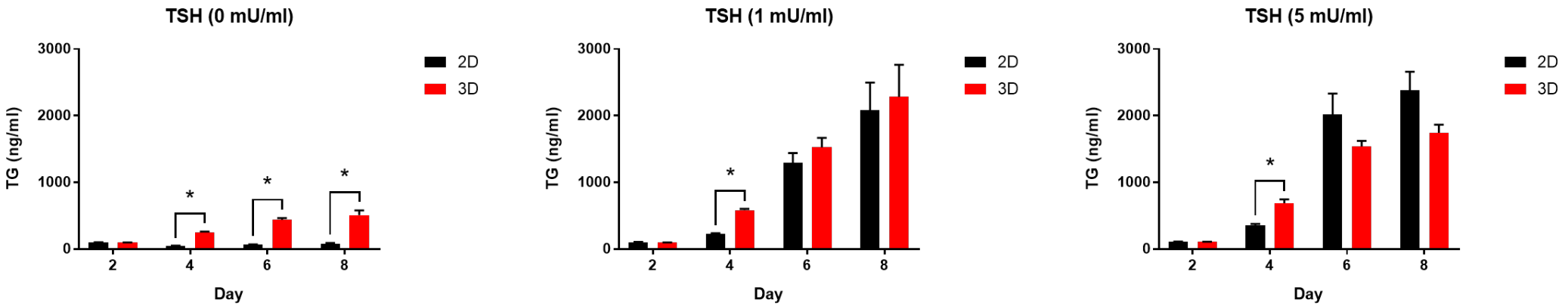
Gene Expression Analysis: 2D vs 3D vs Tissue

Gene	Species
TSHR	Human
TG	Human
TPO	Human
SLC5A5 (NIS)	Human
SLC26A4 (Pendrin)	Human
PAX8	Human
NKX2-1	Human
FOXE1	Human
DUOX1	Human
DUOX2	Human
DUOXA1	Human
DUOXA2	Human
TBP	Human



- Increased differentiation in a 3D model format
- Model- and TSH-dependent increase in genes regulating thyroid hormone synthesis

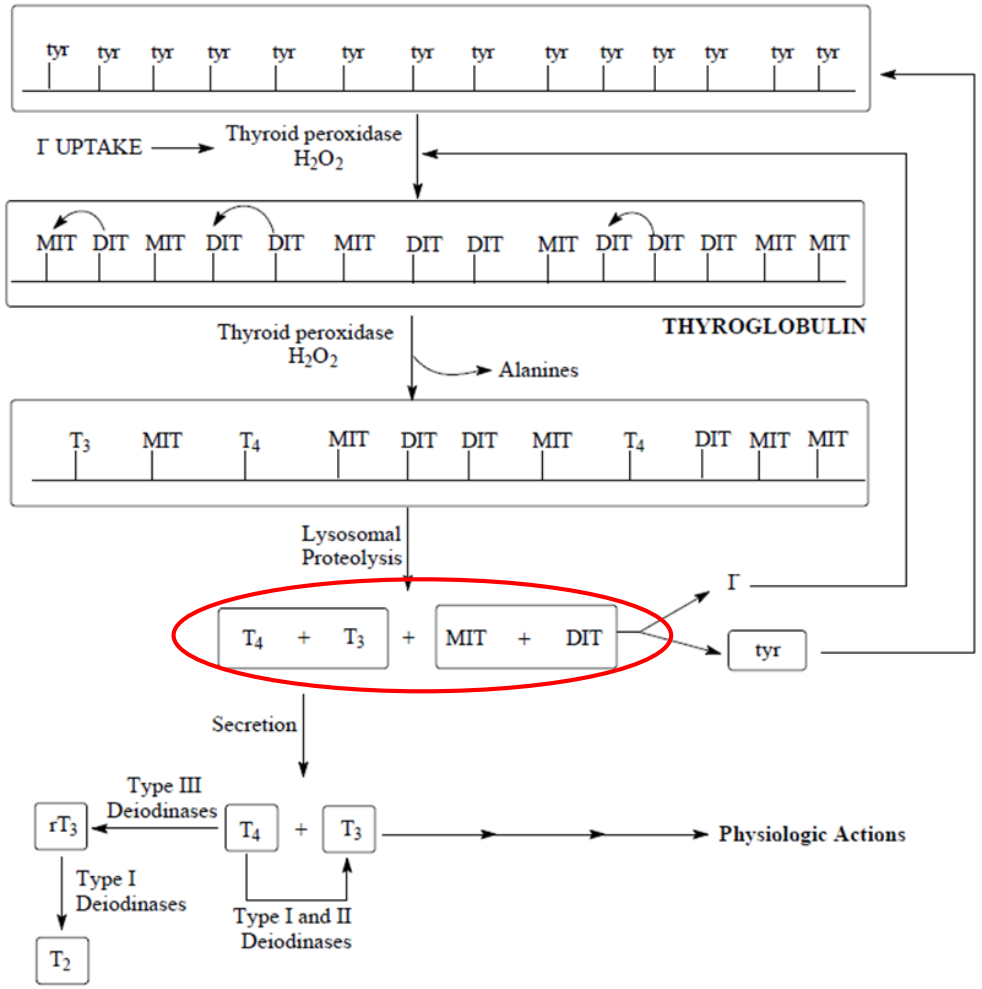
TSH-induced Thyroglobulin Production

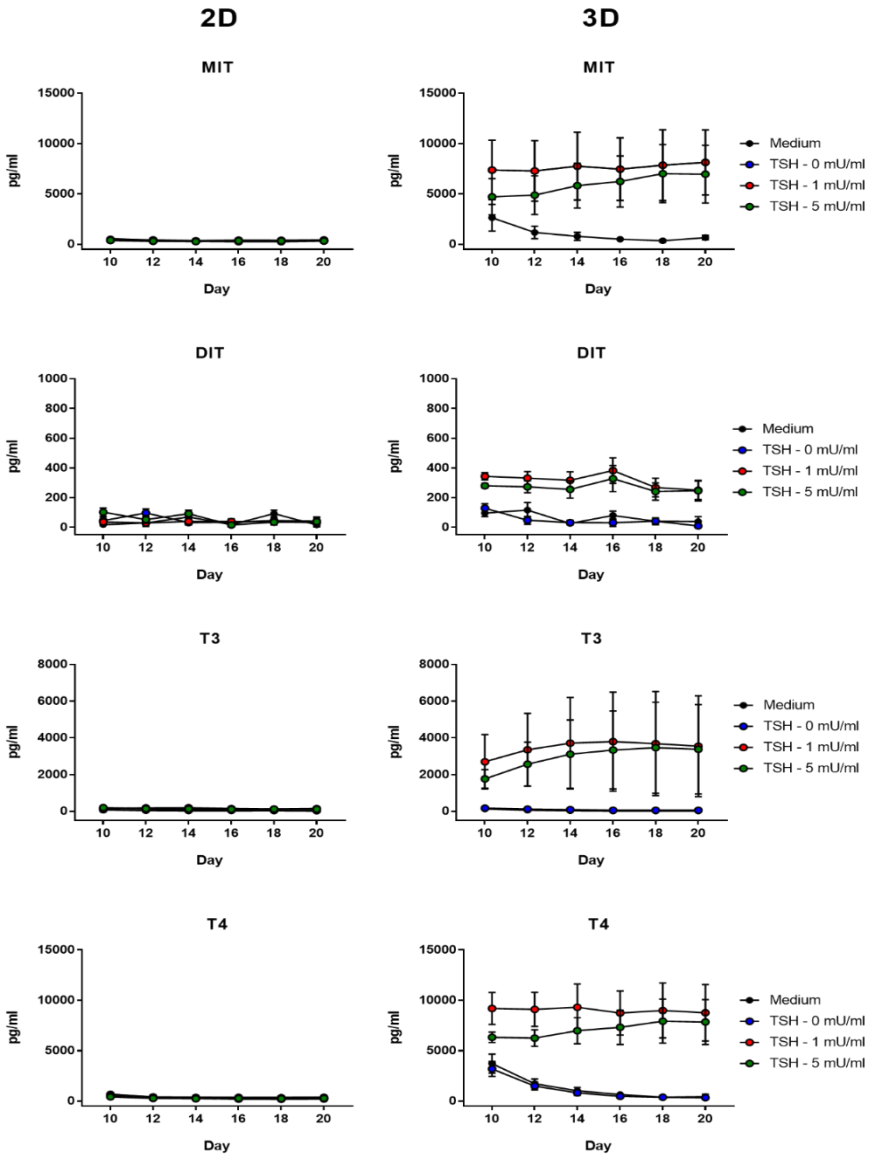


Donor LNH1817005 (n=4)

- Thyroglobulin production increases in a dose- and time-dependent manner
- TSH-dependent induction supports functional TSH receptor (TSHR) activity

Thyroid Hormone Synthesis and Secretion

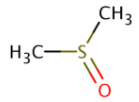
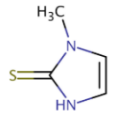
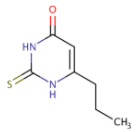
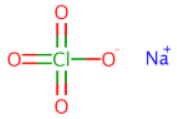
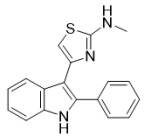
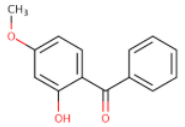




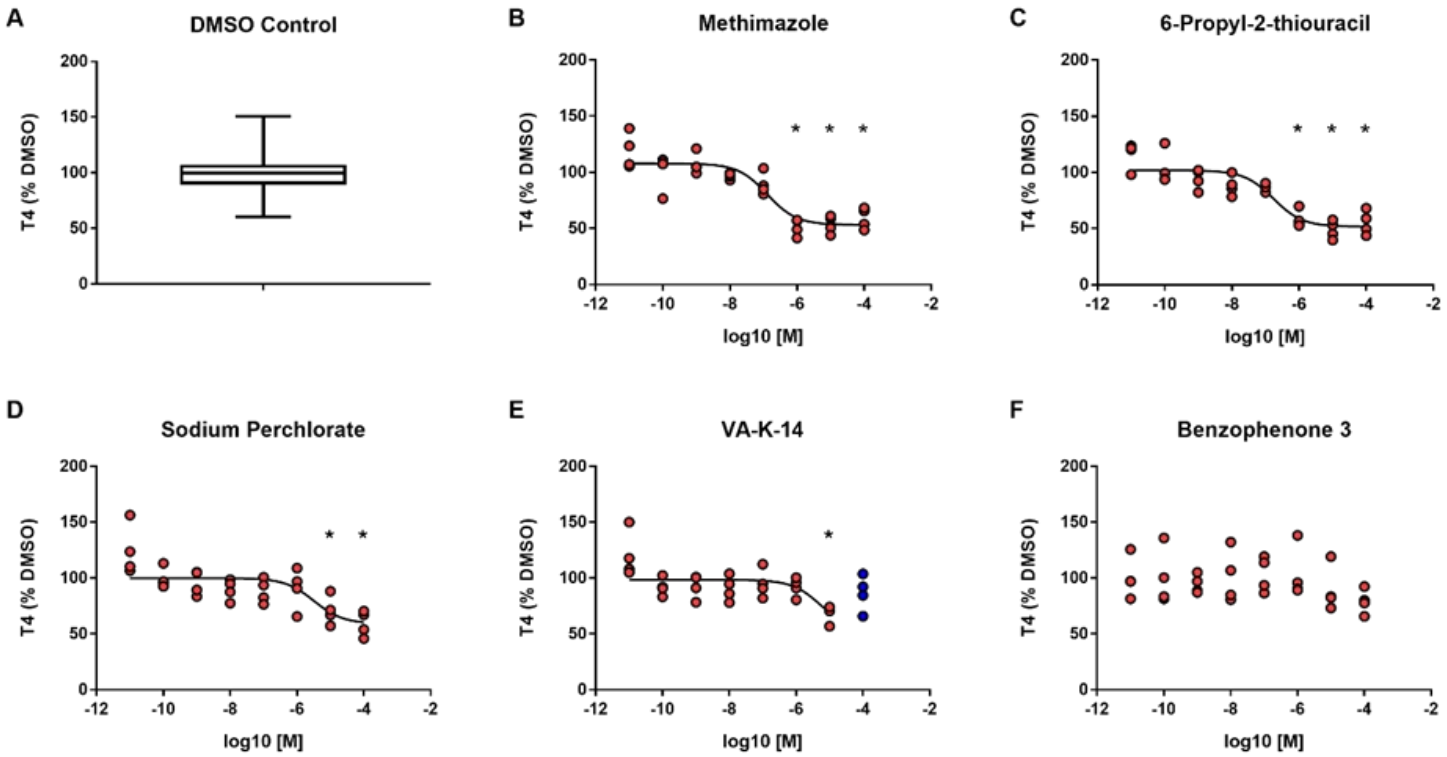
- Data support iodide organification and T4/T3 synthesis exclusively in a 3D model

Donors LNH1722161, LNH1818646, and LNH1910594 (n=3)

Thyroid Disrupting Reference Chemicals

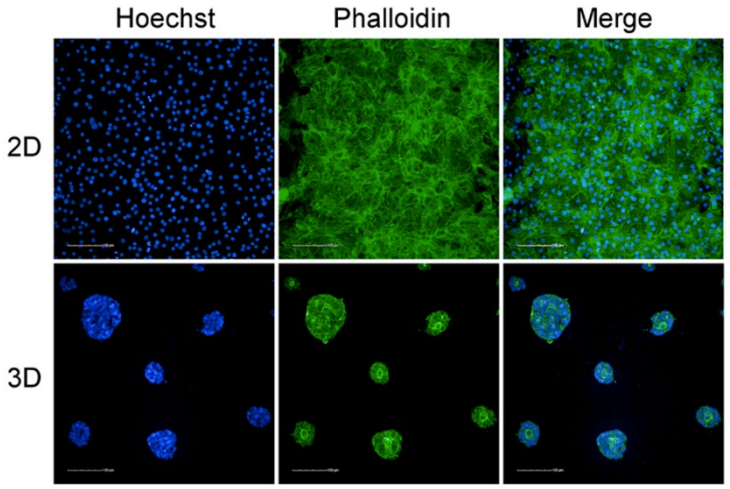
Name	Structure	CASRN	Abbreviation	Target	Classification
Dimethyl Sulfoxide		67-68-5	DMSO	-	Solvent Control
Methimazole		60-56-0	MMI	TPO	TPO Inhibitor
6-Propyl-2-thiouracil		51-52-5	PTU	TPO	TPO Inhibitor
Sodium Perchlorate		7601-89-0	PERC	NIS	NIS Inhibitor
VA-K-14 HCl		1171341-19-7	VAK14	TSHR	TSHR Antagonist
Benzophenone 3		131-57-7	BP3	-	Negative Control

Evaluation of Reference Chemical Inhibition of Thyroid Hormone Synthesis in a 3D Microtissue Culture Model



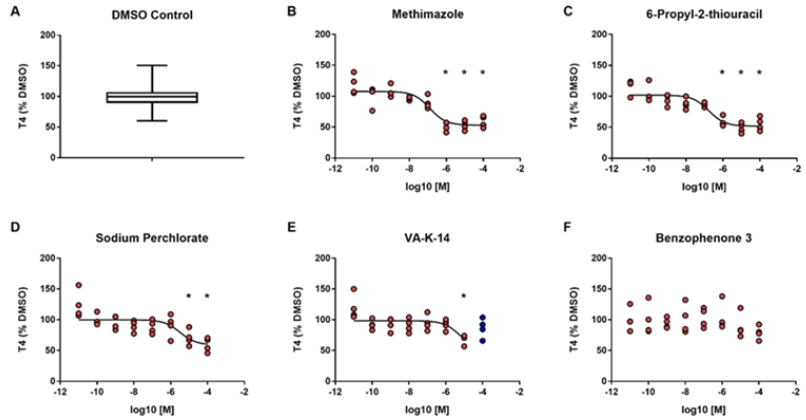
Chemical	IC_{50} (µM)	E_{max} (% T4)	LEC (µM)
Methimazole	0.129	53.0	1
6-Propyl-2-thiouracil	0.172	49.3	1
Sodium Perchlorate	3.23	60.5	10
VA-K-14 HCl	5.61	72.3	10
Benzophenone 3	-	-	-

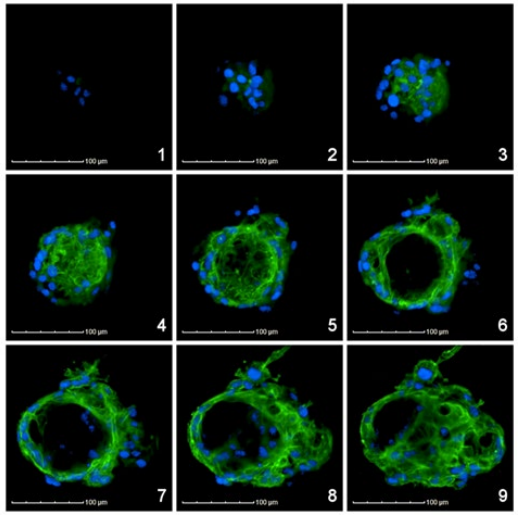
Summary: Clear Advantages to a 3D Human Thyroid Model



Primary human thyrocyte microtissues in an *in vitro* 3D culture model

- **Impact:** An *in vitro* model of the human thyroid that is fully competent in thyroid hormone synthesis
- **Phenotypic Relevance:** Follicular-like morphology, TSHR activation, thyroglobulin synthesis, iodide uptake*, thyroid hormone synthesis and secretion
- **Screening Throughput**: Amenable to medium-throughput (10s-100s), concentration-response testing of HTS prioritized hits
- **Automation Accessible**: Automated liquid handling, acoustic dosing, and high-content imaging
- **Sampling Design**: Cell culture supernatant sampling (Thyroid Hormone and Thyroglobulin) enables kinetic testing and chronic-dosing paradigms
- **Applications**: Drug and chemical testing, organ-on-chip technologies, thyroid disease research and modeling





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