EPA United States Environmental Protection Agency

Development of Novel *In Vitro* Assay Technologies for Human Thyroid Screening

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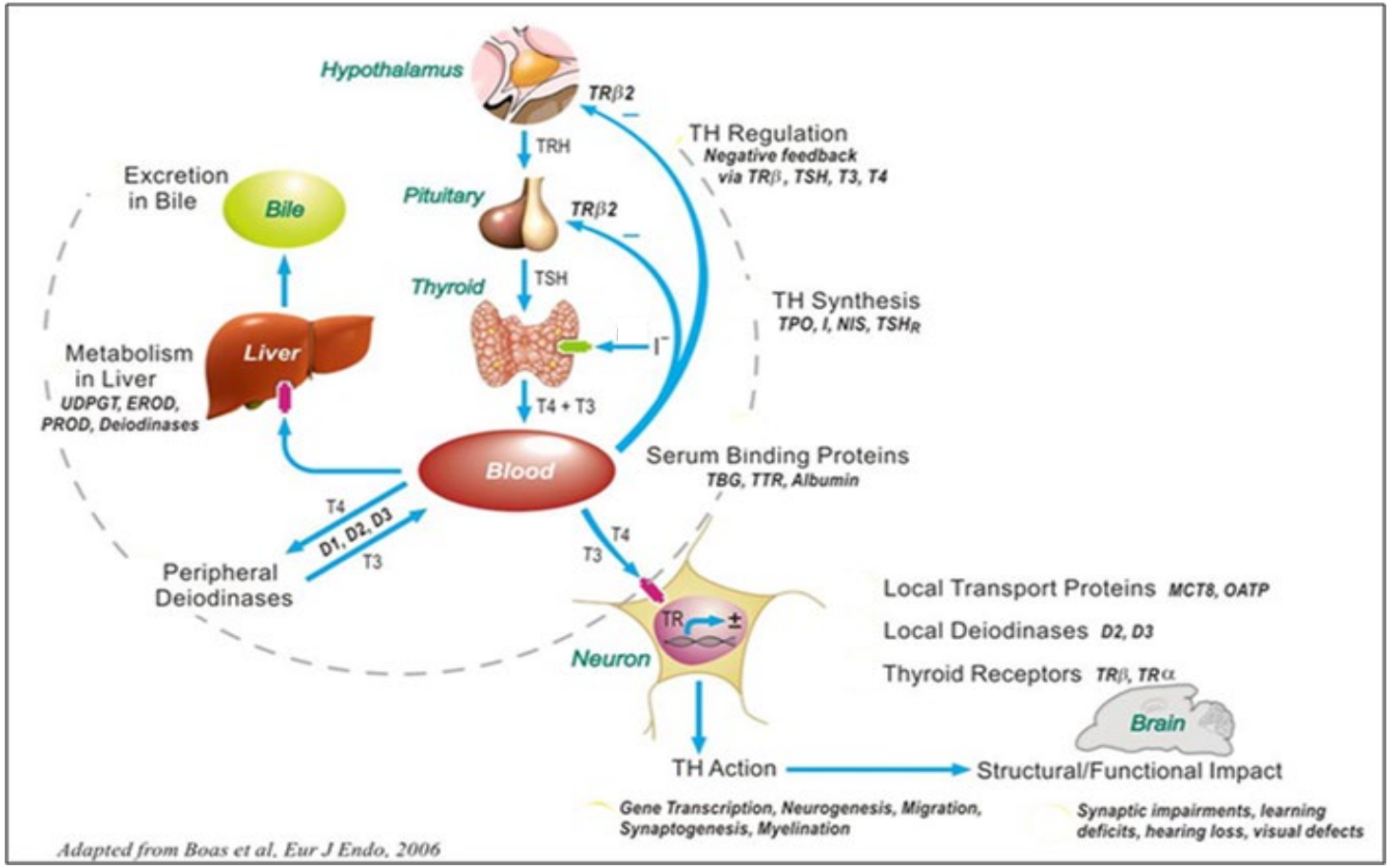
United States Environmental Protection Agency

Outline

- Current Methods for Evaluating Thyroid Toxicity
- Challenges with In Vitro Thyroid Testing: Predicting Thyroid Hormone Disruption from High-throughput Assays
- Development of a Human Thyroid Organotypic Culture Model Assay
- Development of Novel Immortalized Human Thyrocyte Cell Lines

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. 2017. DOI: 10.2779/921523

Thyroid Testing in the 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

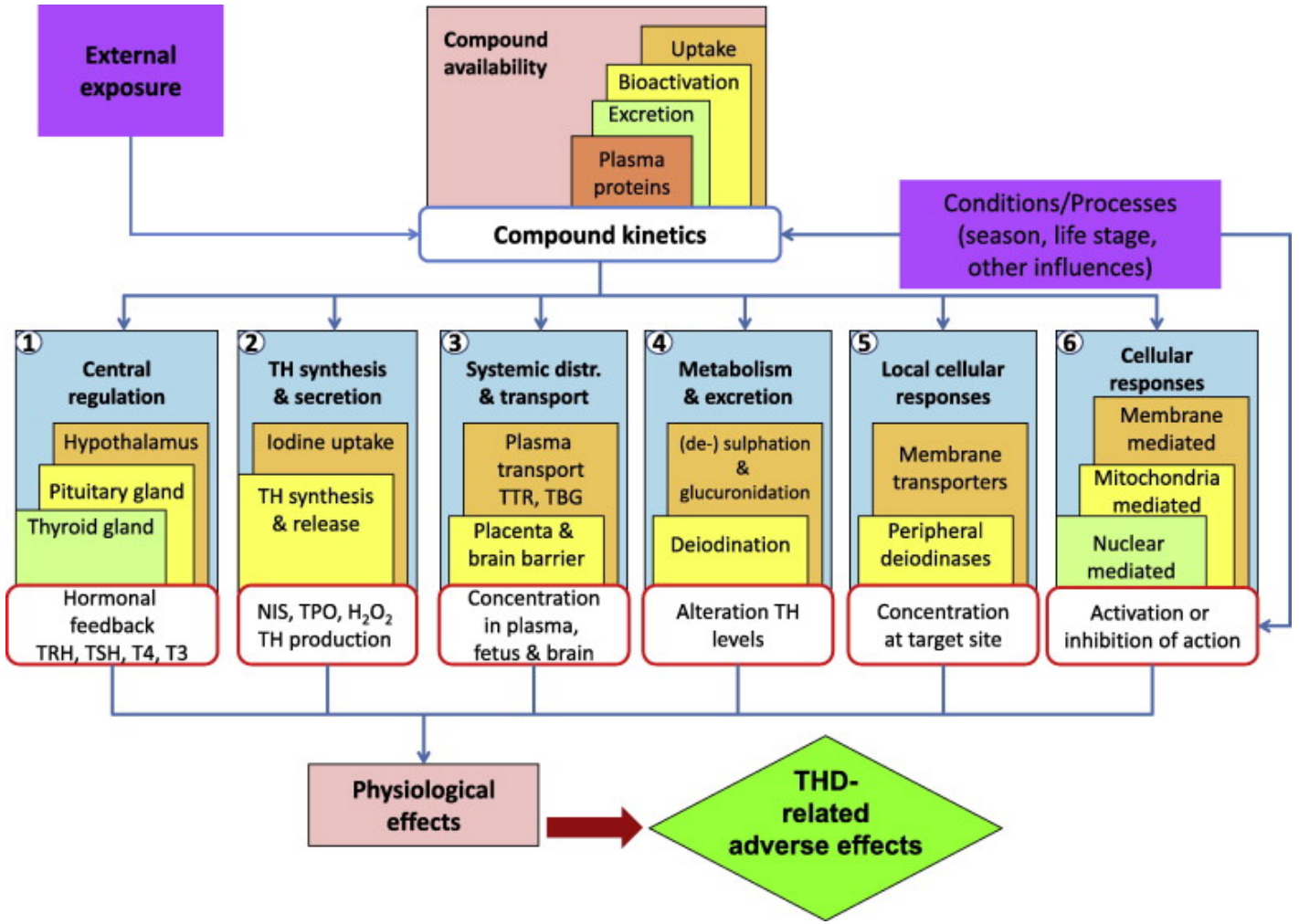
***In vivo* endpoints for thyroid-related endocrine testing in guideline studies**

- Serum T3, 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

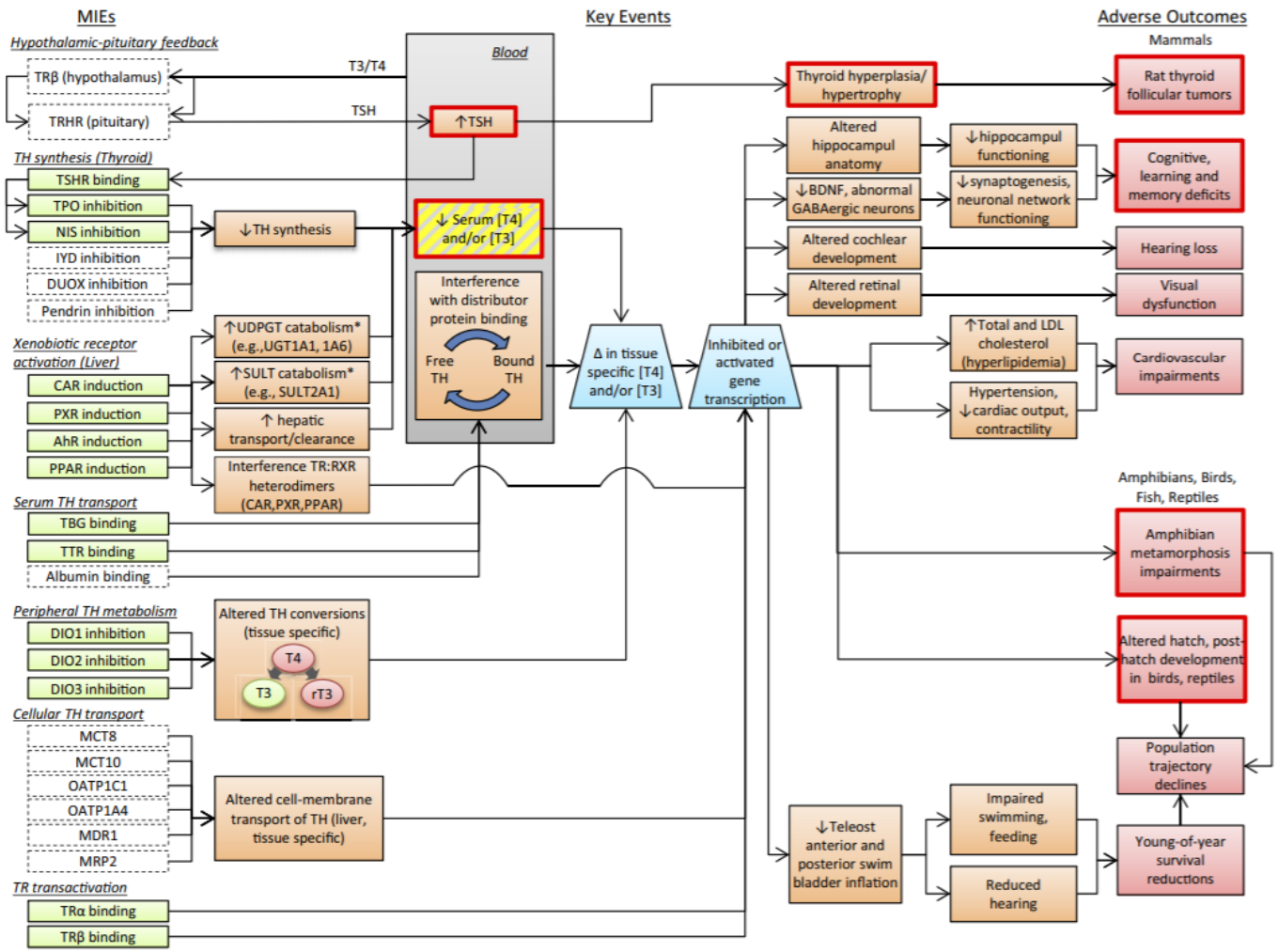


Murk, A. J. *et al.* Mechanism-based testing strategy using in vitro approaches for identification of thyroid hormone disrupting chemicals. *Toxicology in vitro.* (2013).

OECD New Scoping Document on in vitro and ex vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling. (2014).

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).

In Vitro/In Vivo Thyroid Screening: Proposed Thyroid Adverse Outcome Pathway (AOP) Network for Chemical Screening and Assessment



Thyroid AOP network architecture

- Framework to organize and evaluate thyroid data
- Identify data gaps
- Examine causality between key events

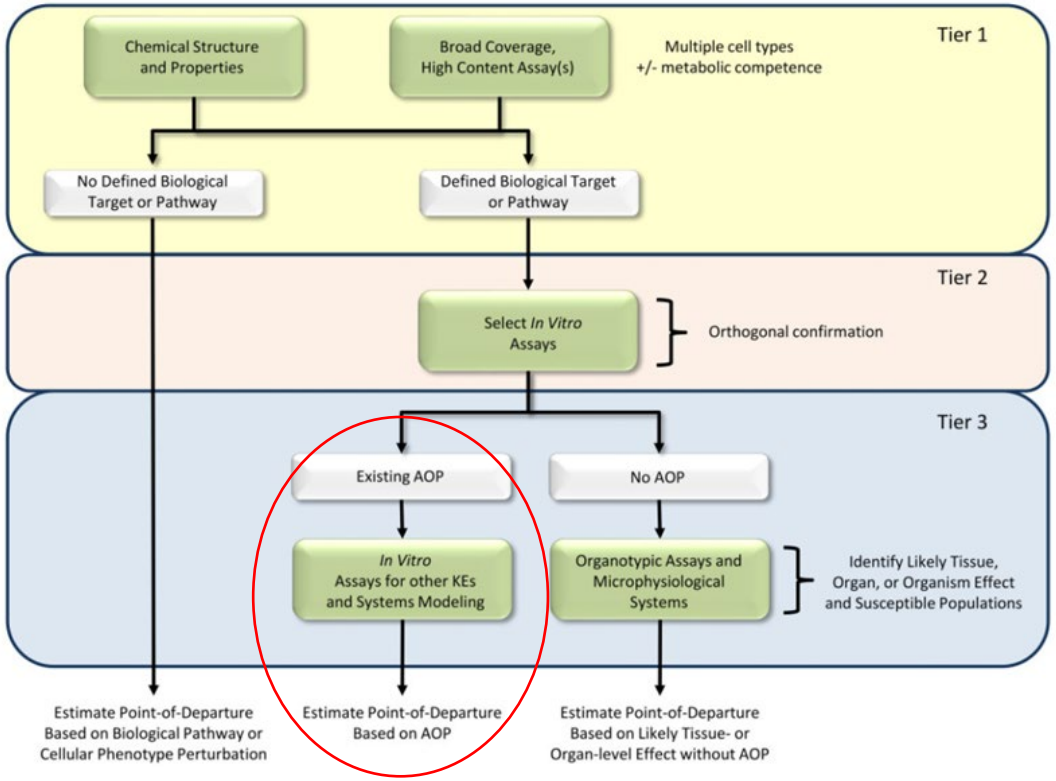
What are the quantitative relationships between MIEs and Key Events that drive adverse outcomes?

What additional bioassay data can be generated to reduce existing data uncertainty and support predictive modeling of thyroid disrupting chemicals?

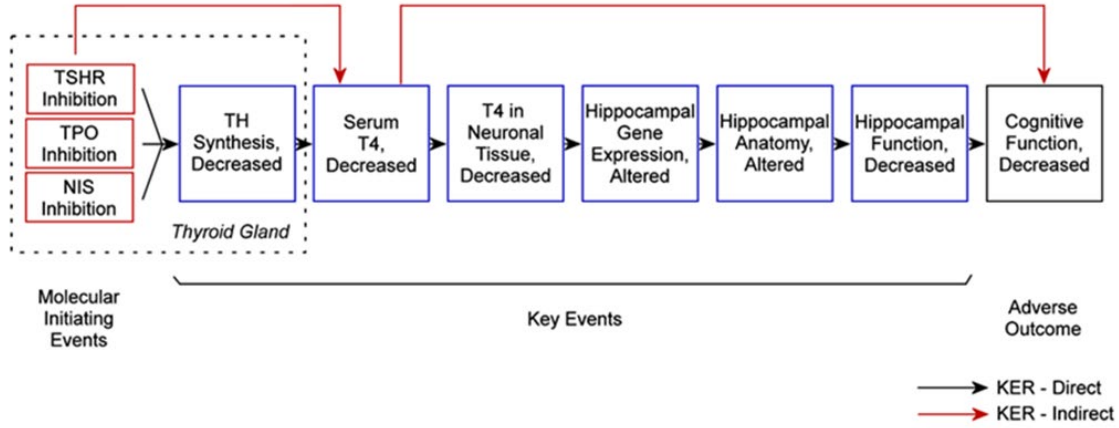
What *in vitro* assay technologies are needed to address key events in the thyroid AOP framework?

Noyes PD, Friedman KP, Browne P, et al. Environ Health Perspect. 2019;127(9):95001. doi:10.1289/EHP5297

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



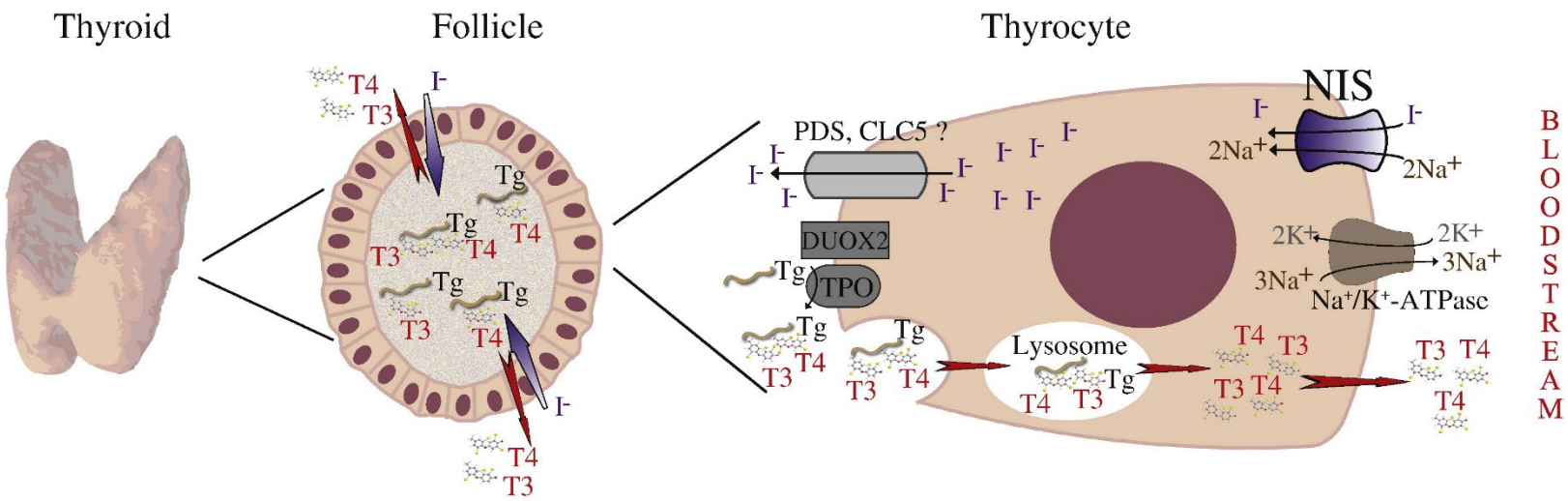
- The uncertainty surrounding the specificity of active chemicals identified in thyroid gland-related screens and the relevance to phenotypic effects on *in vivo* human thyroid hormone synthesis are notable data gaps for hazard identification of thyroid disrupting chemicals (TDCs).
- Additional data linking MIE effects to thyroid hormone synthesis and secretion could support predictive modeling of TDCs for related adverse outcomes.



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

Thomas RS et al. Toxicol Sci. 2019;169(2):317–332.

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

- Structure-function relationship: 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

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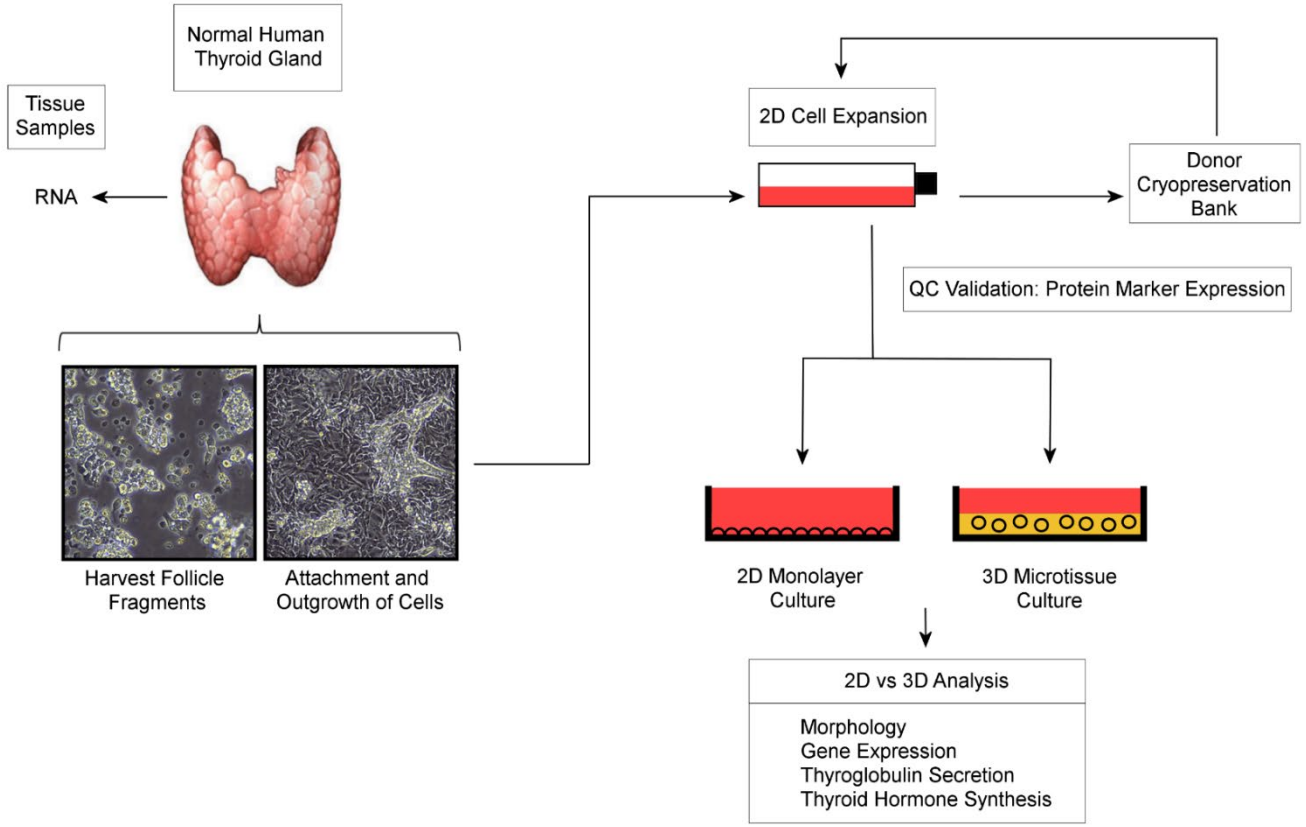
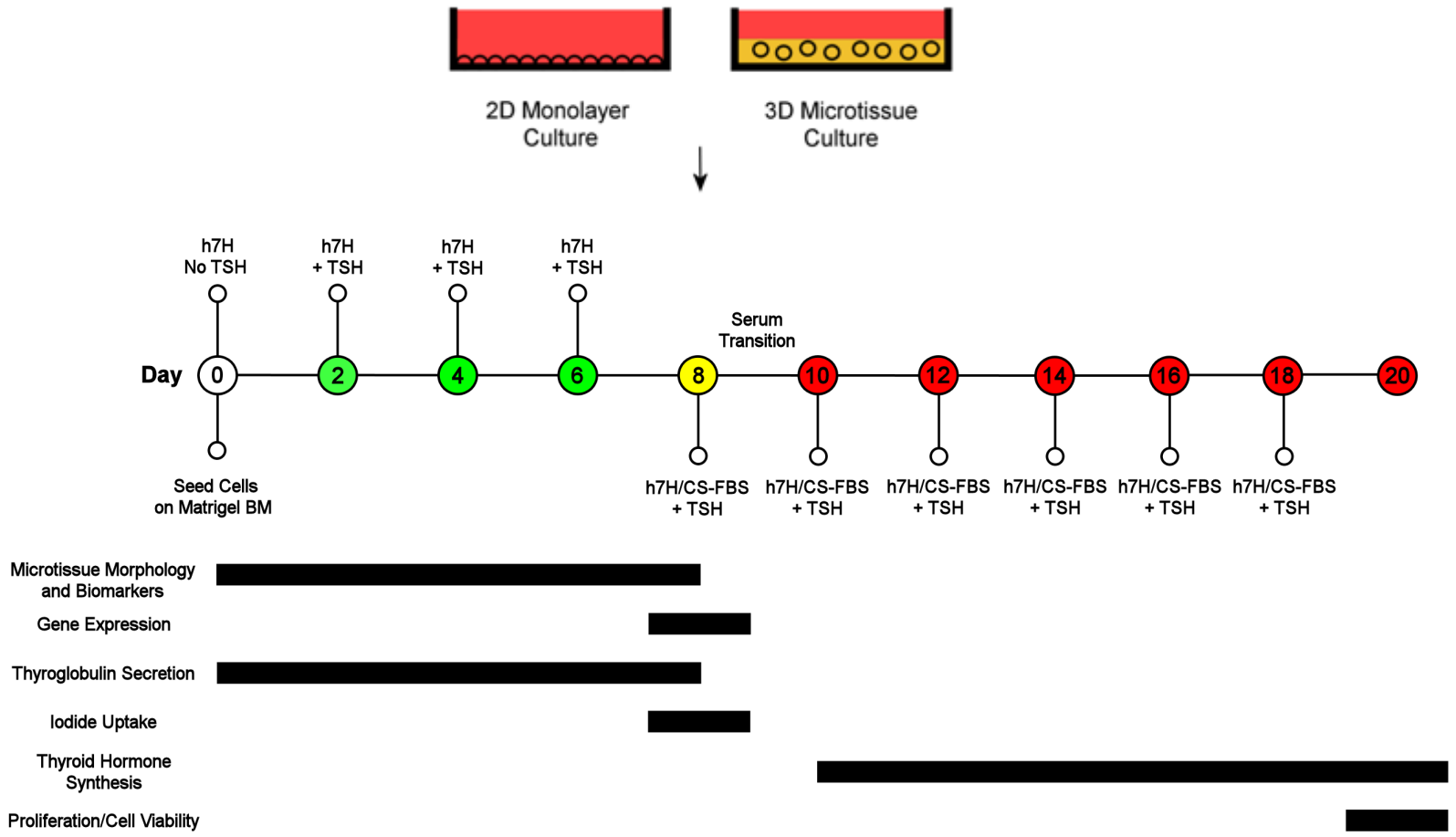


Study objective: Develop a medium-throughput organotypic screening assay comprised of reconstructed human thyroid microtissues to quantitatively evaluate the disruptive effects of chemicals on thyroid hormone synthesis and secretion.

Deisenroth et al, 2020, Toxicol Sci, 174 (1), 63-78

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

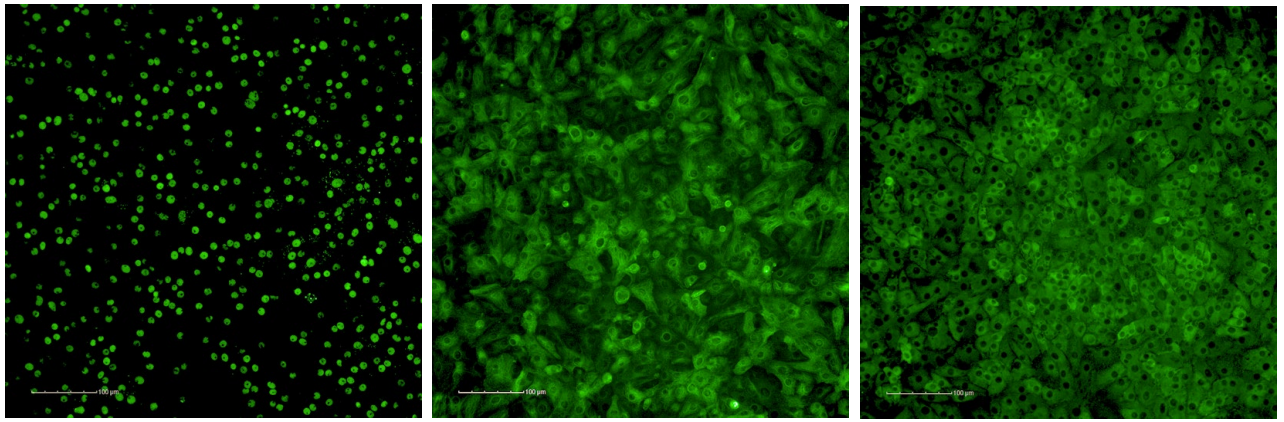
- Institute of Regenerative Medicine develops innovative or novel uses of donor tissues and organs through sound scientific and clinical research
- **Thyroid:** Procurement, digestion, expansion, cryopreservation

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

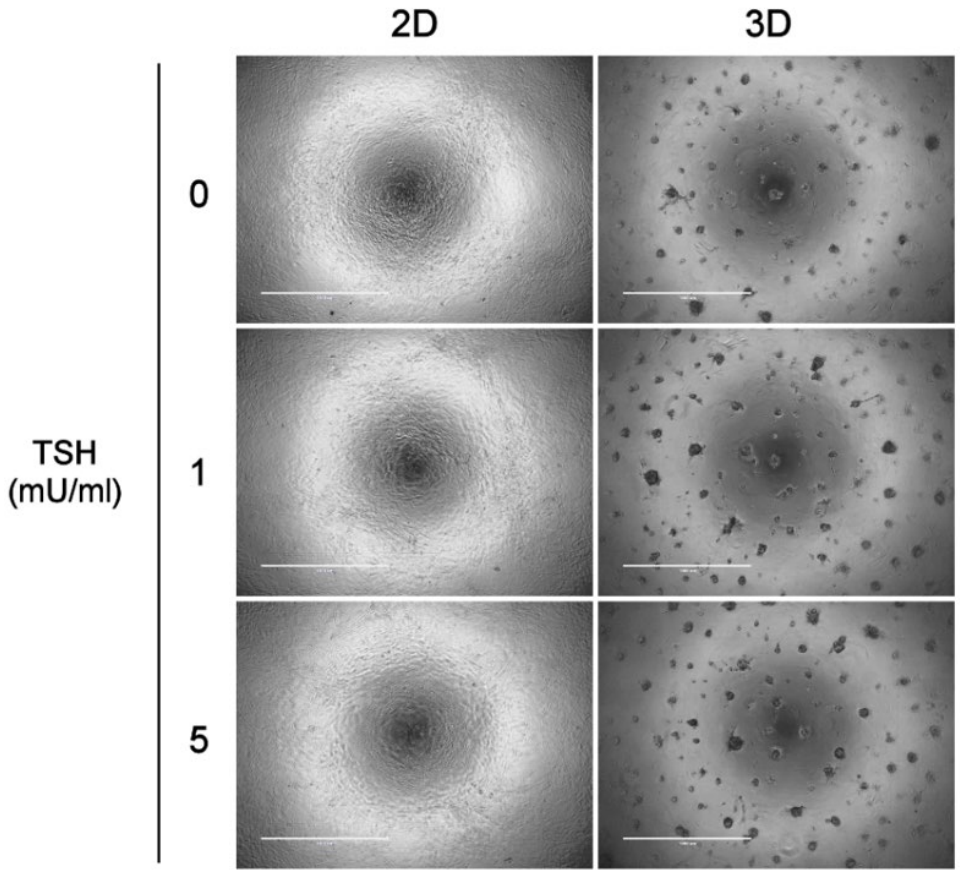


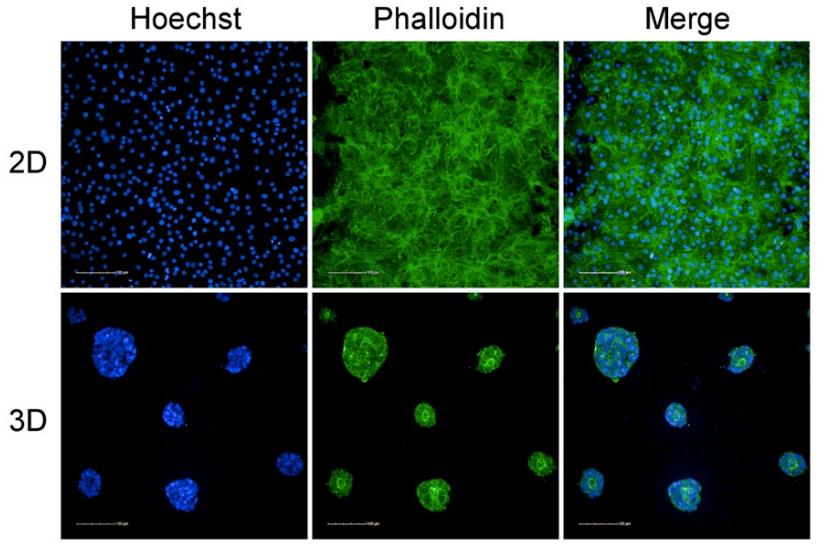
	IgG			IgG, kappa			NKX2-1			KRT7			TG		
Biomarker	**% POS**	**SEM**	**N**	**% POS**	**SEM**	**N**	**% POS**	**SEM**	**N**	**% POS**	**SEM**	**N**	**% POS**	**SEM**	**N**
NKX2-1	1.91	0.50	6	-	-	-	95.18	1.74	6	-	-	-	-	-	-
KRT7	-	-	-	0.30	0.14	6	-	-	-	90.52	2.47	6	-	-	-
TG	-	-	-	1.93	1.31	6	-	-	-	-	-	-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

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

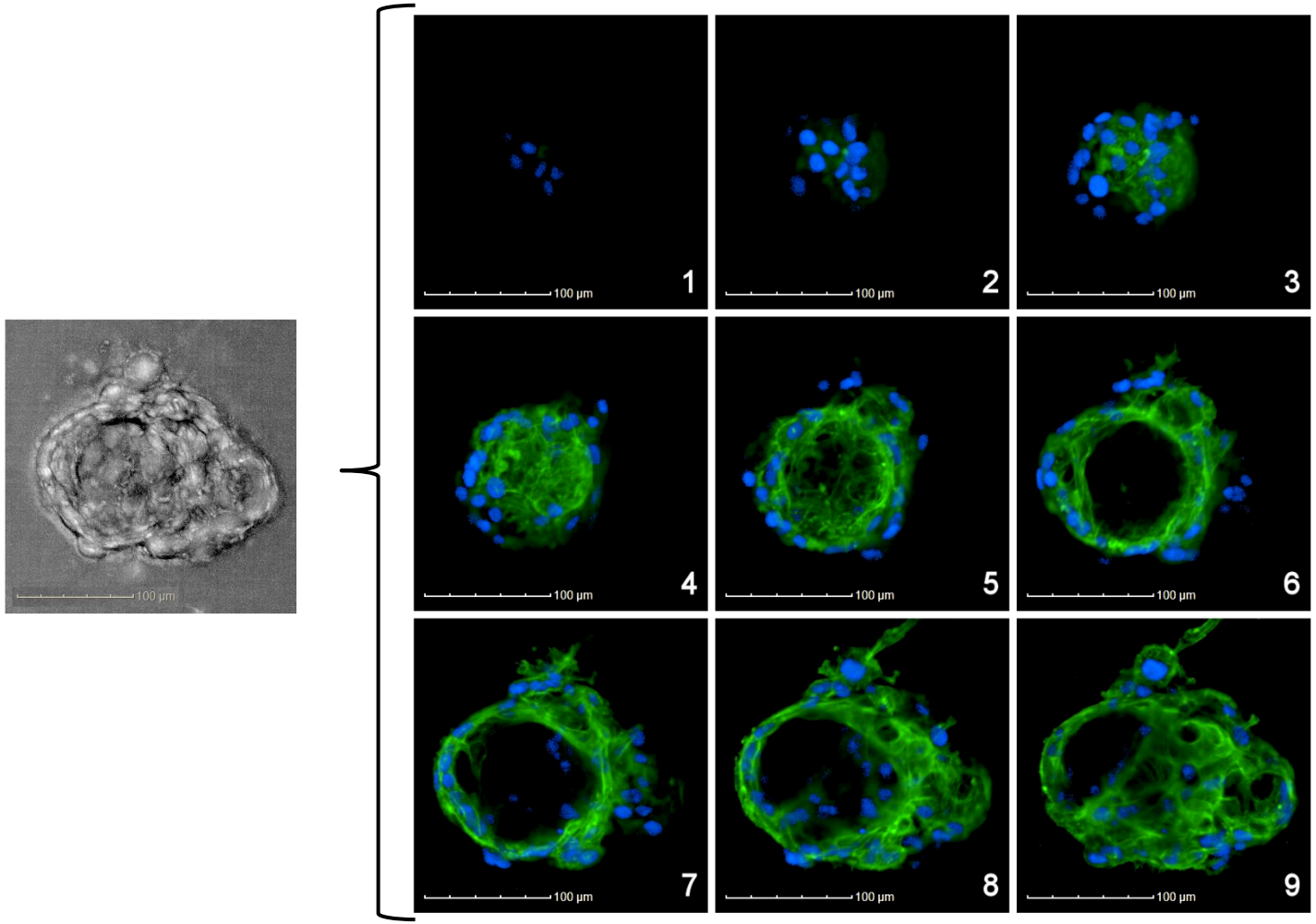
Table 3. Biomarker Image Cytometry. The cell-level frequency of IgG isotype controls (α-Mouse IgG kappa and α-Rat IgG), NK2 Homeobox 1 (NKX2-1), Keratin 7 (KRT7), and Thyroglobulin (TG) staining were quantitatively evaluated by high-content imaging across 6 independent human donors for verification of 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





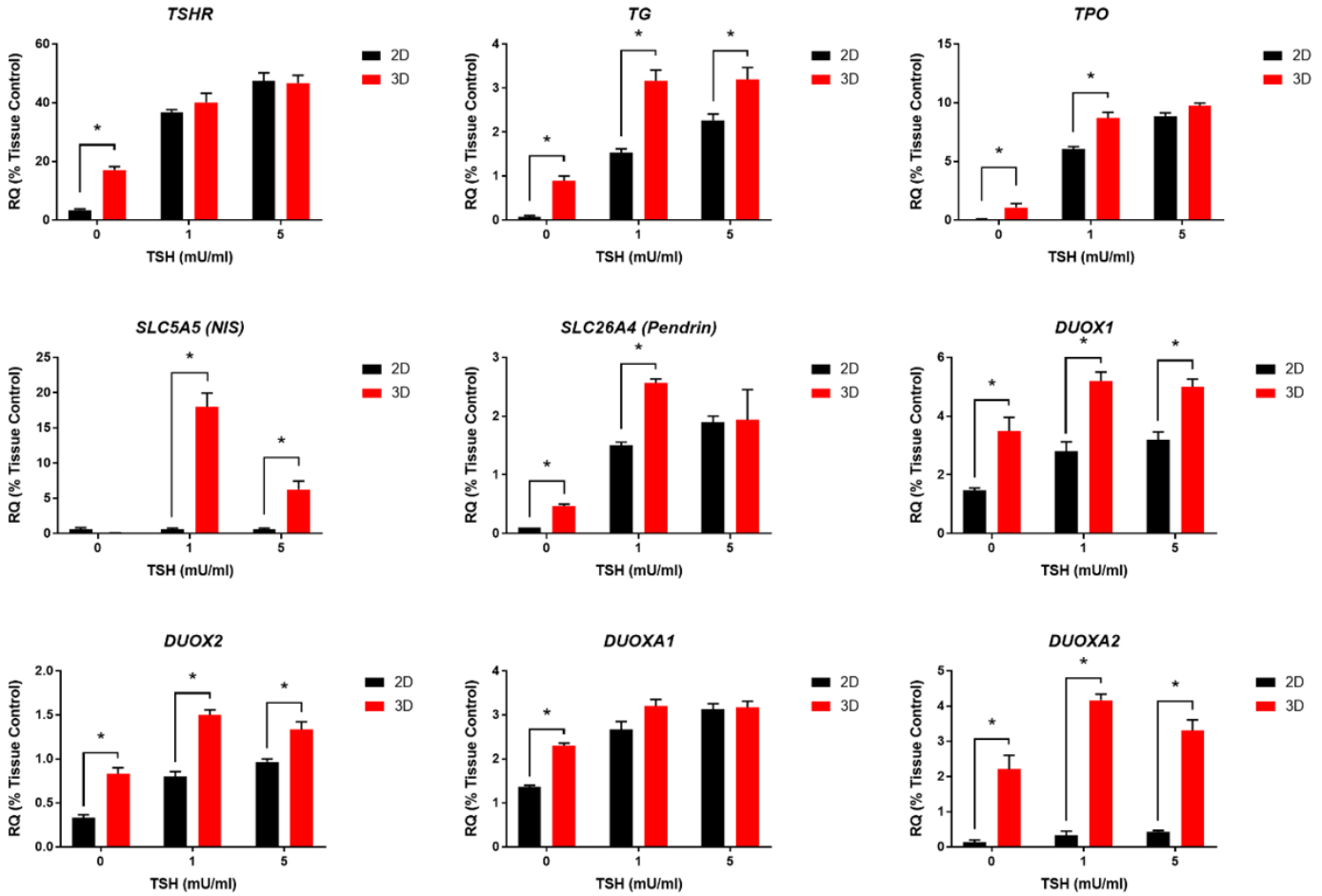
Donor Thyrocyte Characterization: Follicle-like Architecture



Donor LNH1722161: Confocal series of 3D microtissue.

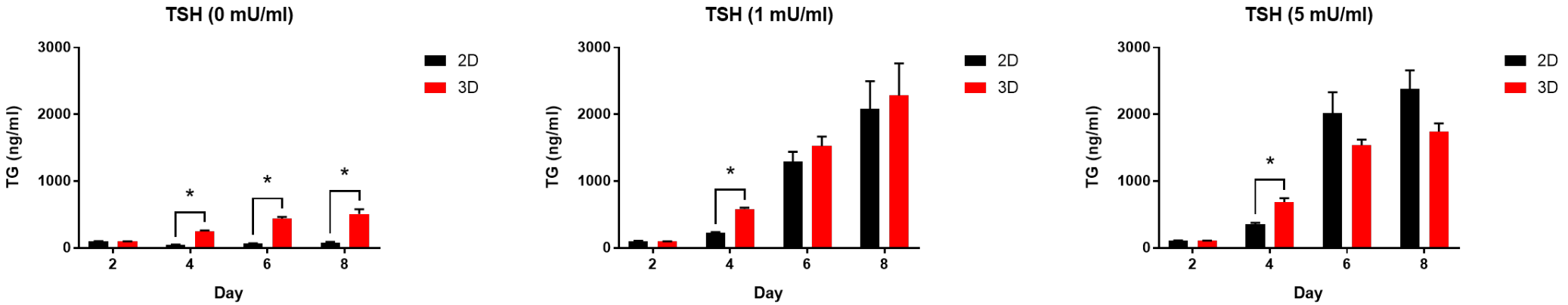
Gene Expression Analysis: 2D vs 3D vs Tissue

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

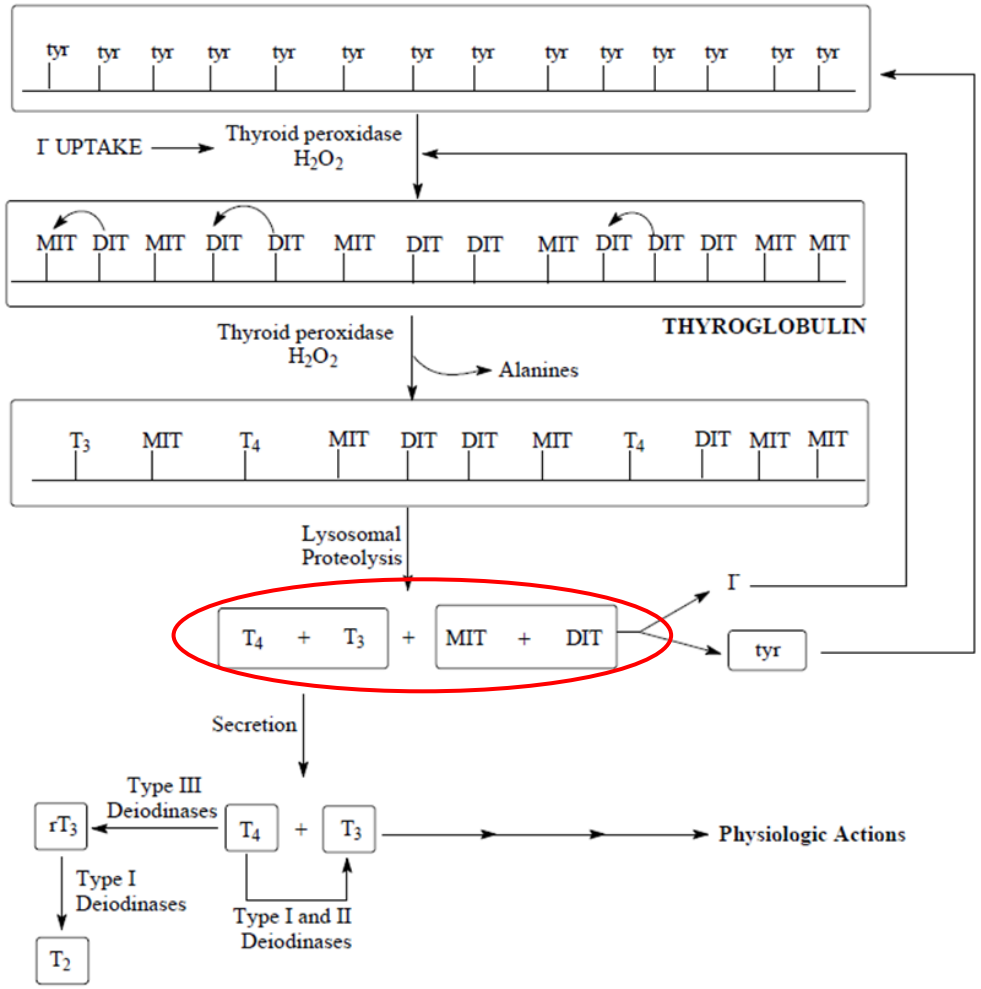
TSH-induced Thyroglobulin Production



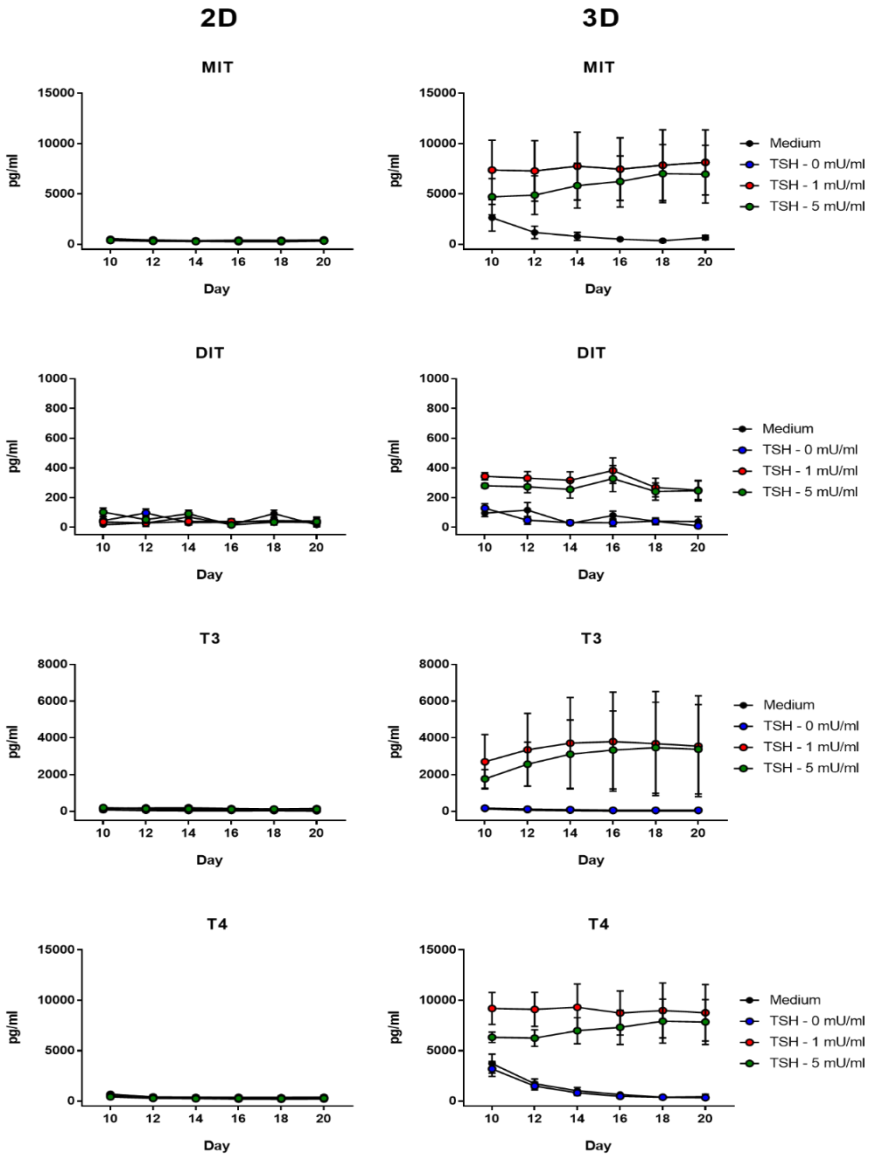
Donor LNH1817005 (n=4)

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

Thyroid Hormone Synthesis and Secretion

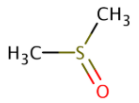
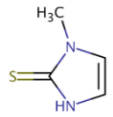
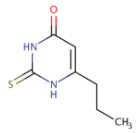
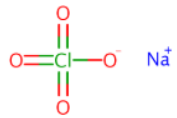
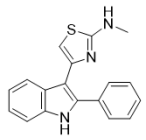
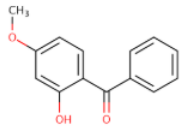


- Data support iodide organification and T4/T3 synthesis exclusively in a 3D model

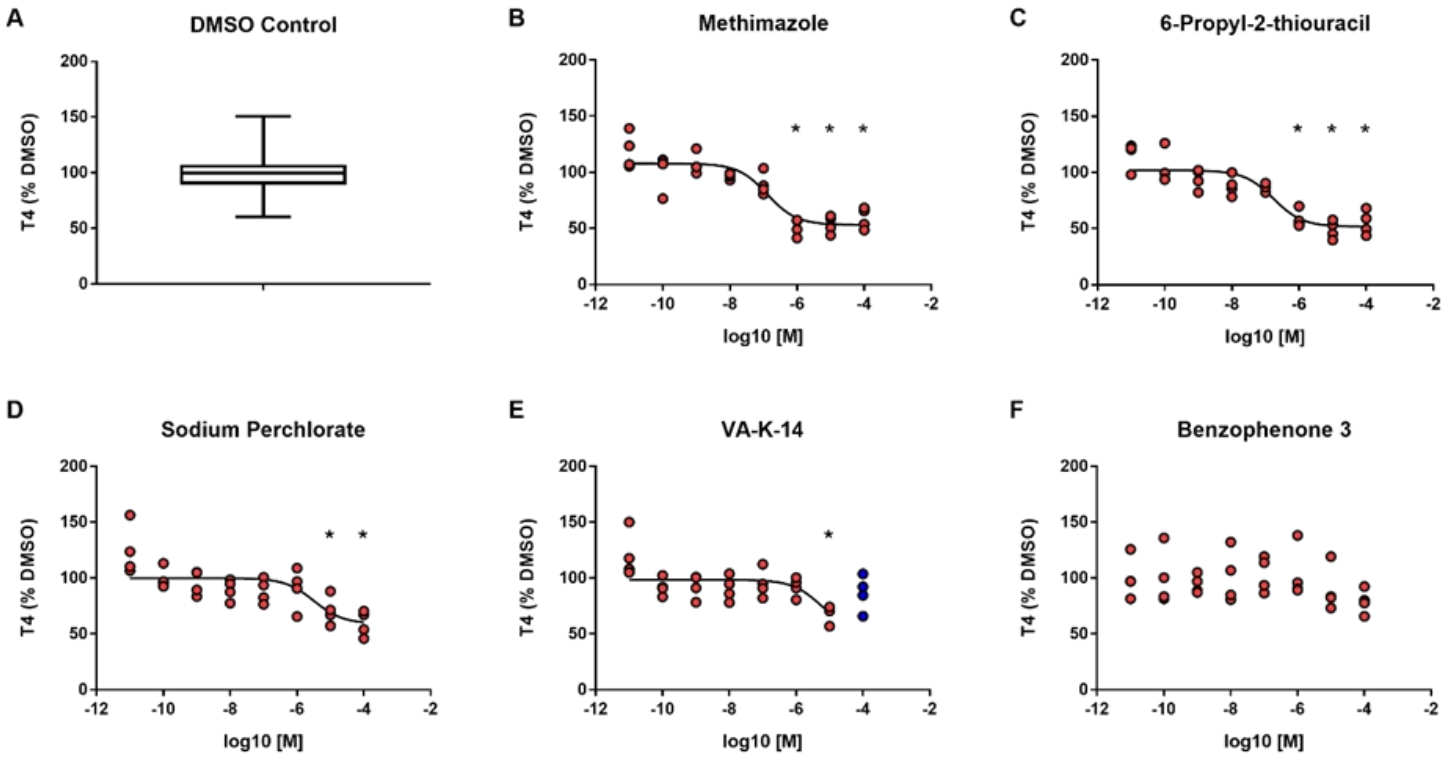


Donors LNH1722161, LNH1818646, and LNH1910594 (n=3)

Thyroid Disrupting Reference Chemicals

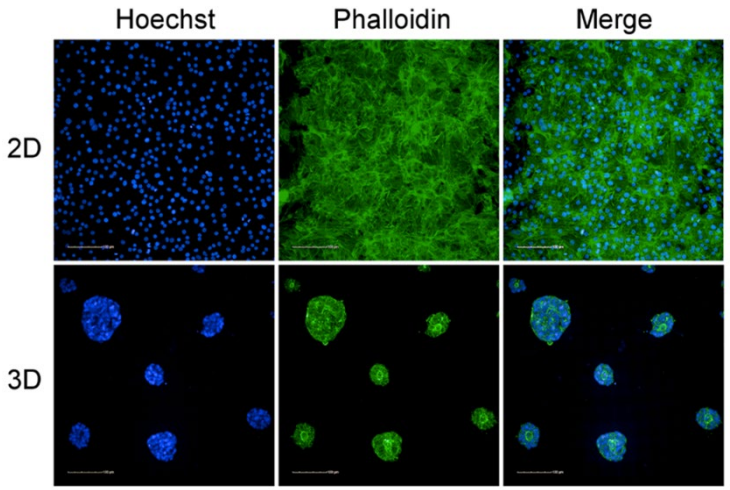
Name	Structure	CASRN	Target	Classification
Dimethyl Sulfoxide		67-68-5	-	Solvent Control
Methimazole		60-56-0	TPO	TPO Inhibitor
6-Propyl-2-thiouracil		51-52-5	TPO	TPO Inhibitor
Sodium Perchlorate		7601-89-0	NIS	NIS Inhibitor
VA-K-14 HCl		1171341-19-7	TSHR	TSHR Antagonist
Benzophenone 3		131-57-7	-	Negative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

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

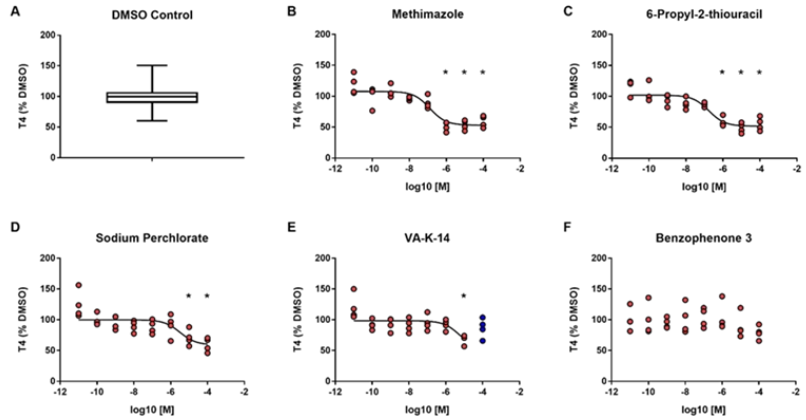


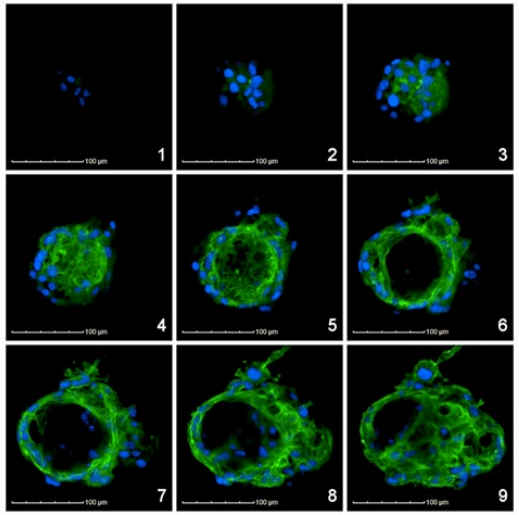
Chemical	IC_{50} (µM)	E_{max} (% T4)	LEC (µM)
Methimazole	0.129	53.0	1
6-Propyl-2-thiouracil	0.172	49.3	1
Sodium Perchlorate	3.23	60.5	10
VA-K-14 HCl	5.61	72.3	10
Benzophenone 3	-	-	-

Summary: 3D Human Thyroid 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



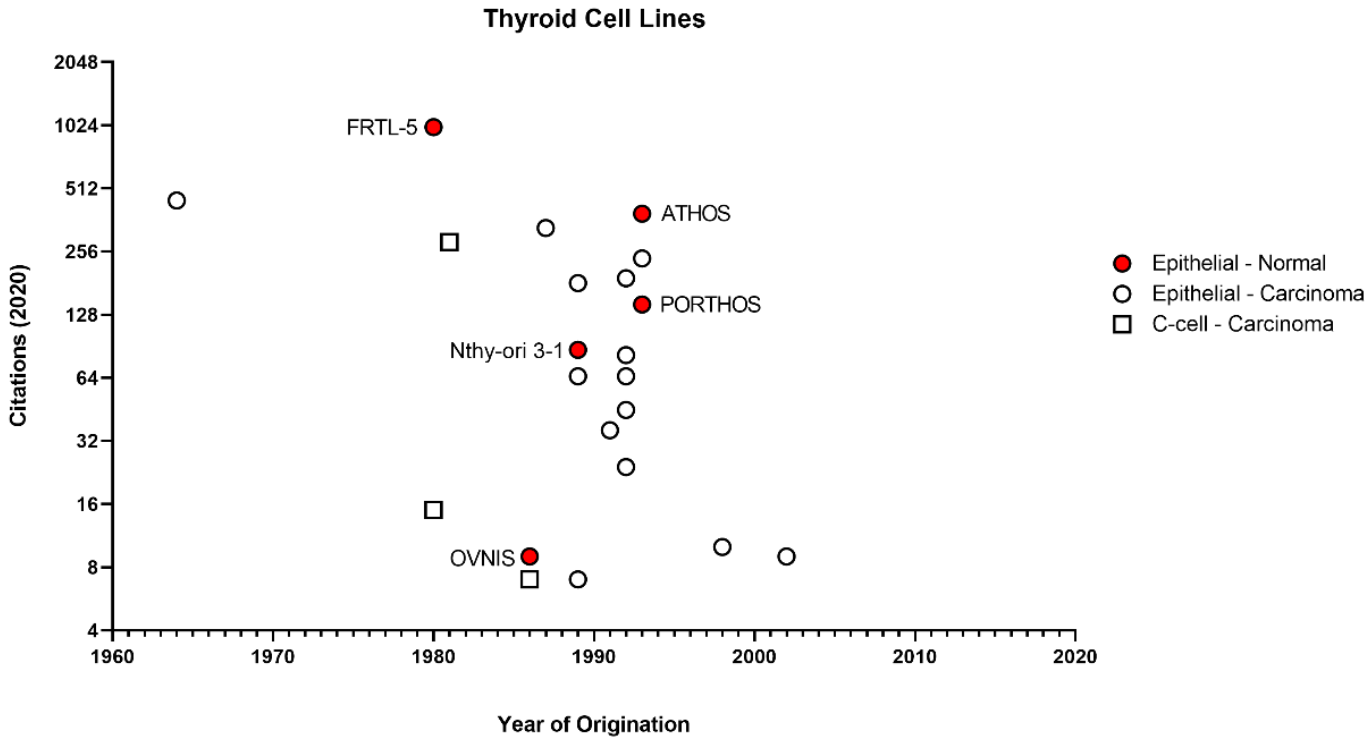


Expanding Assay Transfer and Reproducibility: Identification of Human Thyrocyte Cell Lines

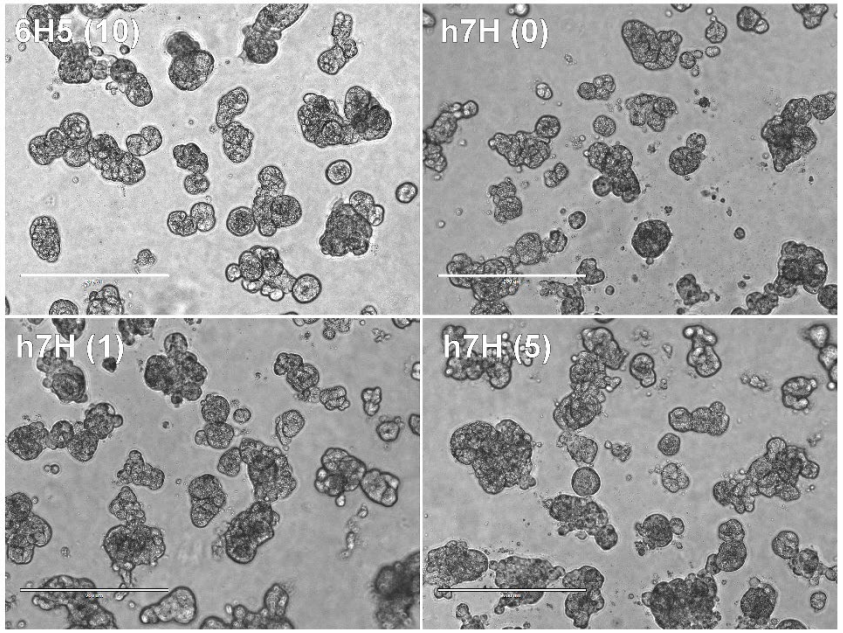
Question: Is there an alternative cell type to recapitulate thyroid hormone synthesis in the 3D microtissue assay?

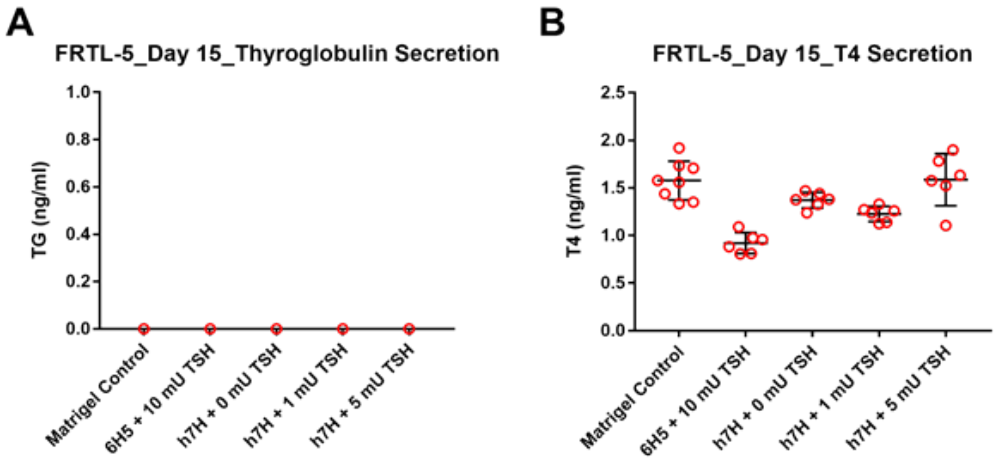
Options:

1. Primary thyrocytes
2. Immortalized thyrocyte cell line
3. Stem cell-derived thyrocytes
4. Thyroid tumor-derived cell line



Thyrocyte Cell Lines May Not Retain Key Functional Features of Primary Thyrocytes





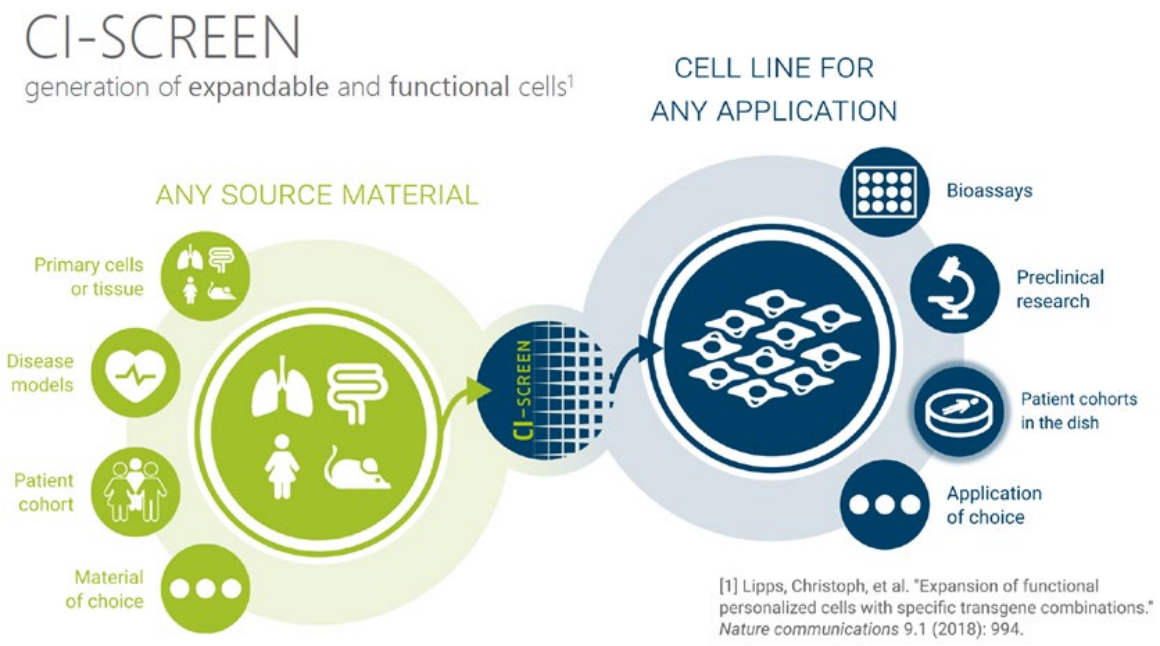
Cell Line: Fischer Rat Thyrocyte Line (FRTL-5) most commonly used in thyroid-related studies. (est 1980)

Evaluation: Medium formulations for rat (6H5) and human (h7H) in 3D culture model. ±TSH (0-10 mU/ml).

Results: Self-organized morphology consistent with primary human thyrocytes, but Thyroglobulin production (TG) and Thyroid Hormone synthesis (T4) not detected.

Conclusion: Immortalized cell lines age! Loss of phenotypic stability is a common observation for thyroid-derived epithelial cells. Genetic drift and adaptation to cell culture conditions over many population doublings can result in physiological deviation from the tissue of origin.

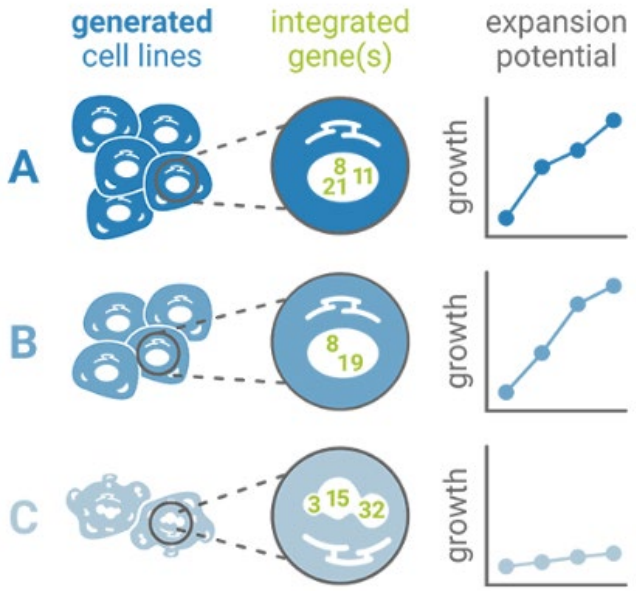
Development of Novel Immortalized Human Thyrocyte Cell Lines

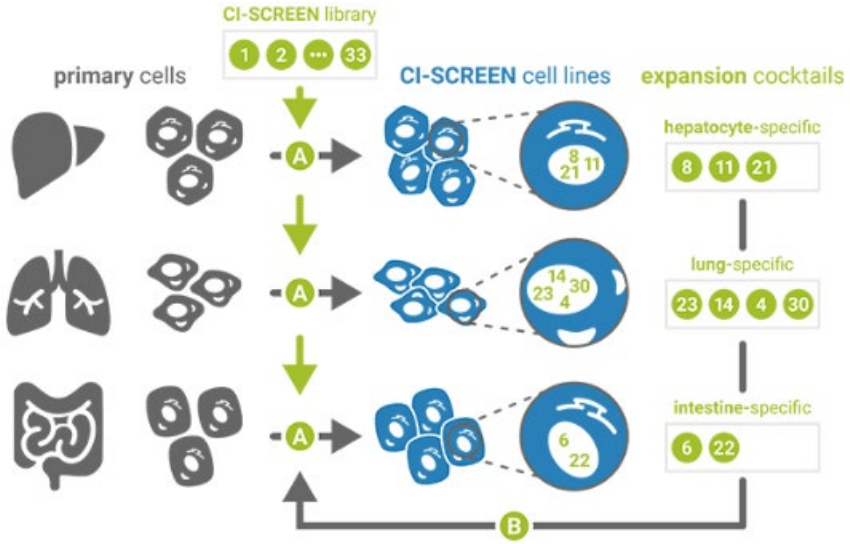


Objective: Create a novel early passage immortalized human thyrocyte line that retains functions of primary thyrocytes.

InSCREENeX CI-SCREEN Technology

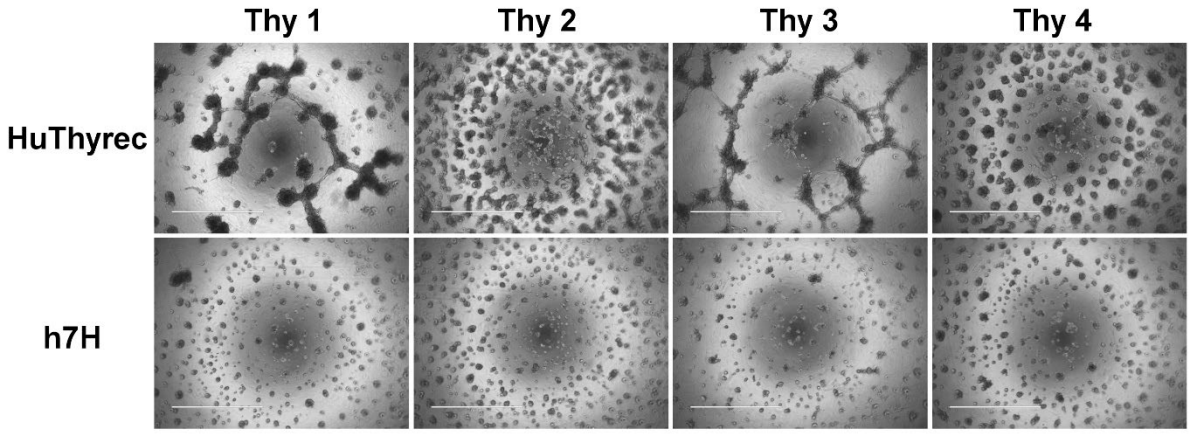
- The CI-SCREEN technology builds upon a unique optimized gene library of 33 genes associated with apoptosis, cell cycle control and stemness.
- After transduction with the library, all generated cell lines are screened for self-selected clonal isolates with expansion capacity consistent with immortalization.

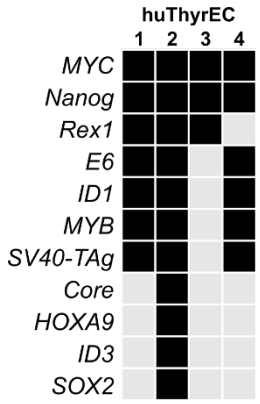




- The CI-SCREEN technology identifies cell type-specific expansion cocktails.
- Screening a high number of immortalized cell lines identifies immortalization gene sets that are cell type specific.

Characterization of Novel Immortalized Human Thyrocyte Cell Lines



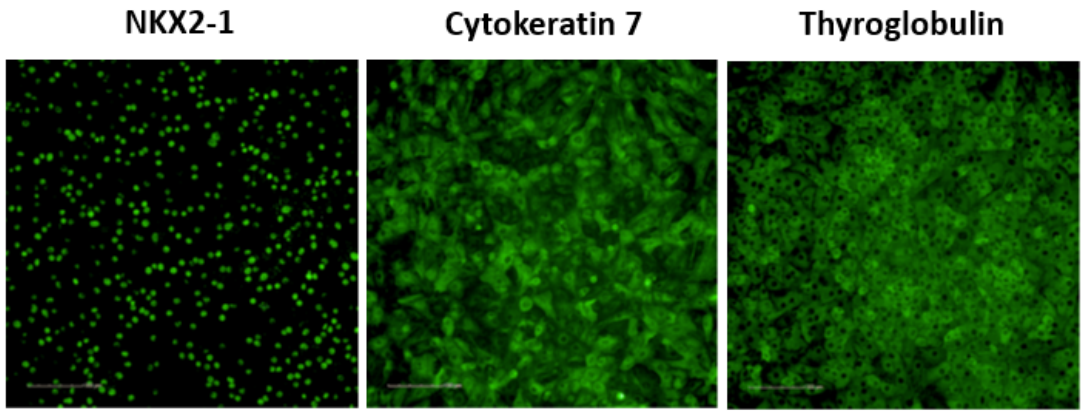


Evaluation

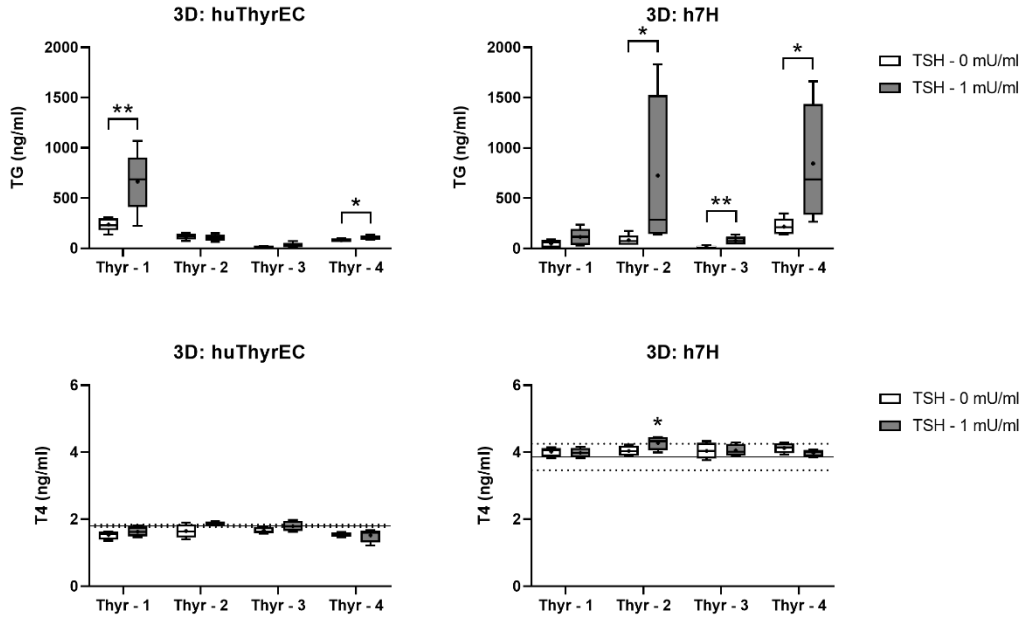
- Thyroid biomarker expression
- Karyotype
- Proliferation rates
- Morphology in 2D and 3D culture formats
- TSH Receptor function
- Thyroglobulin expression
- Thyroid hormone synthesis

Findings

- Novel immortalized human thyrocyte cell lines retain many key morphological and functional features of primary thyrocytes suitable for select phenotypes.



	Thy 1 (A5-1)		Thy 2 (A1)		Thy 3 (AA-1)		Thy 4 (AA-2)	
Biomarker	h7H	huThyrec	h7H	huThyrec	h7H	huThyrec	h7H	huThyrec
NKX2-1	96.5	96.7	98.5	98.5	93.9	92.3	99.1	98.8
KRT7	74.9	91.7	79	93.2	72	89.2	61.8	81.7
TG	4.3	1.8	0.5	1.9	2.3	0.6	2.9	0.6
	Mean expression frequency [% Pos] of 3 technical replicates							



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