Inter-laboratory Prevalidation Study of the 3D Human Thyroid Microtissue Assay

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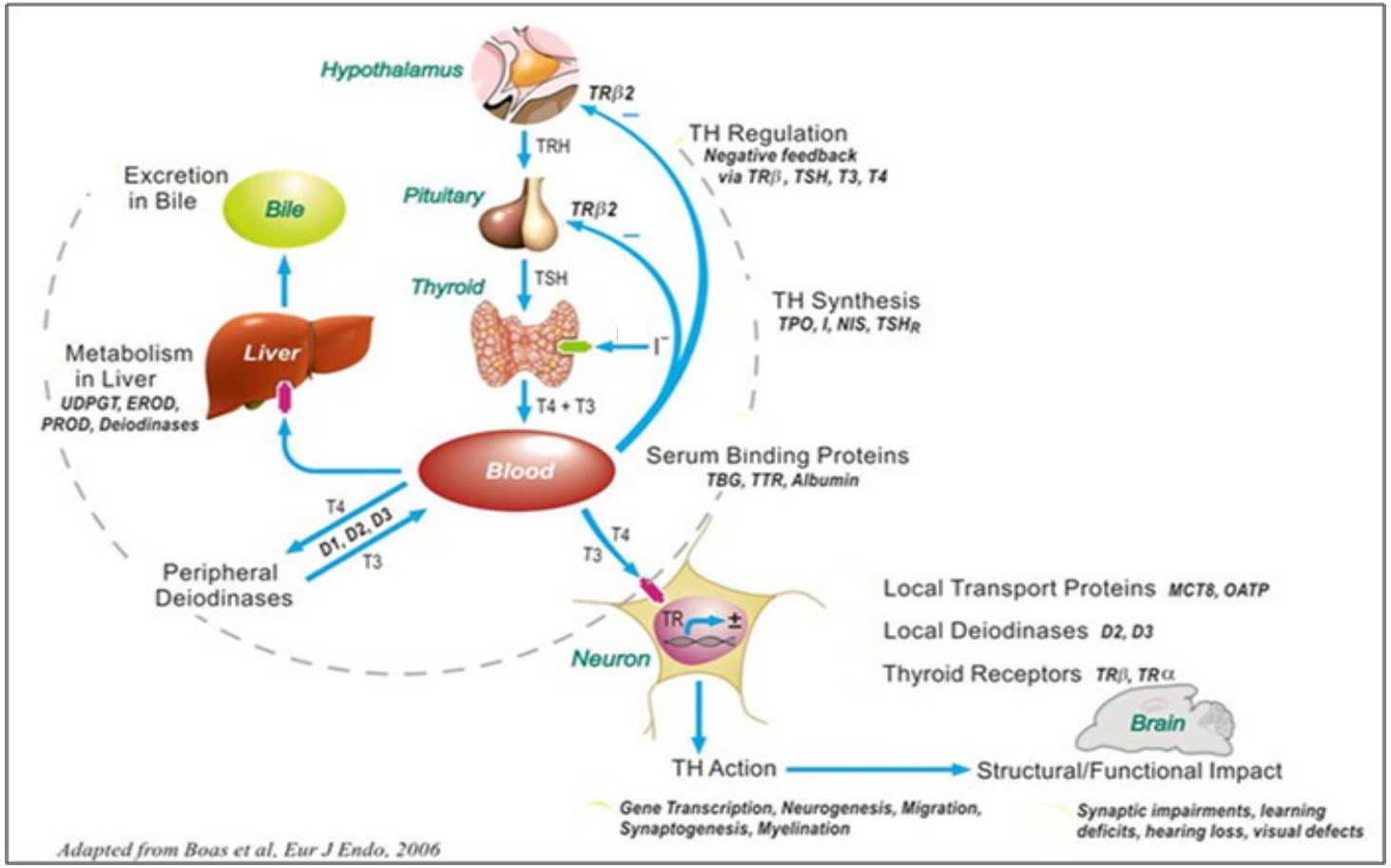
Agenda

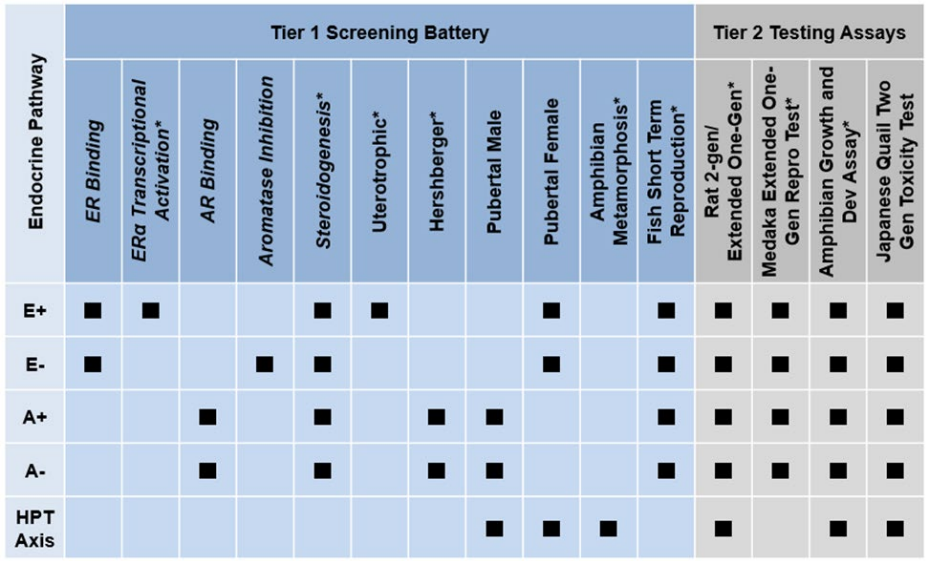
- **Introduction:** Thyroid toxicology and testing strategies.
- **New Approach Methods**: In vitro thyroid test method innovations and regulatory status.
- **Prevalidation Study Proposal**: Complete a prevalidation study that establishes reagents, methods, reference chemicals, and intra- and inter-laboratory performance of the assay.
- **Discussion**: Possible ICCVAM support and guidance - forming a validation management group, staff resources, study coordination, NTP chemistry contract to source test compounds and send blinded samples to the participating labs, report writing, etc.

Endocrine Toxicology: Thyroid Disruption



- 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 are associated with thyroid dysfunction.

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

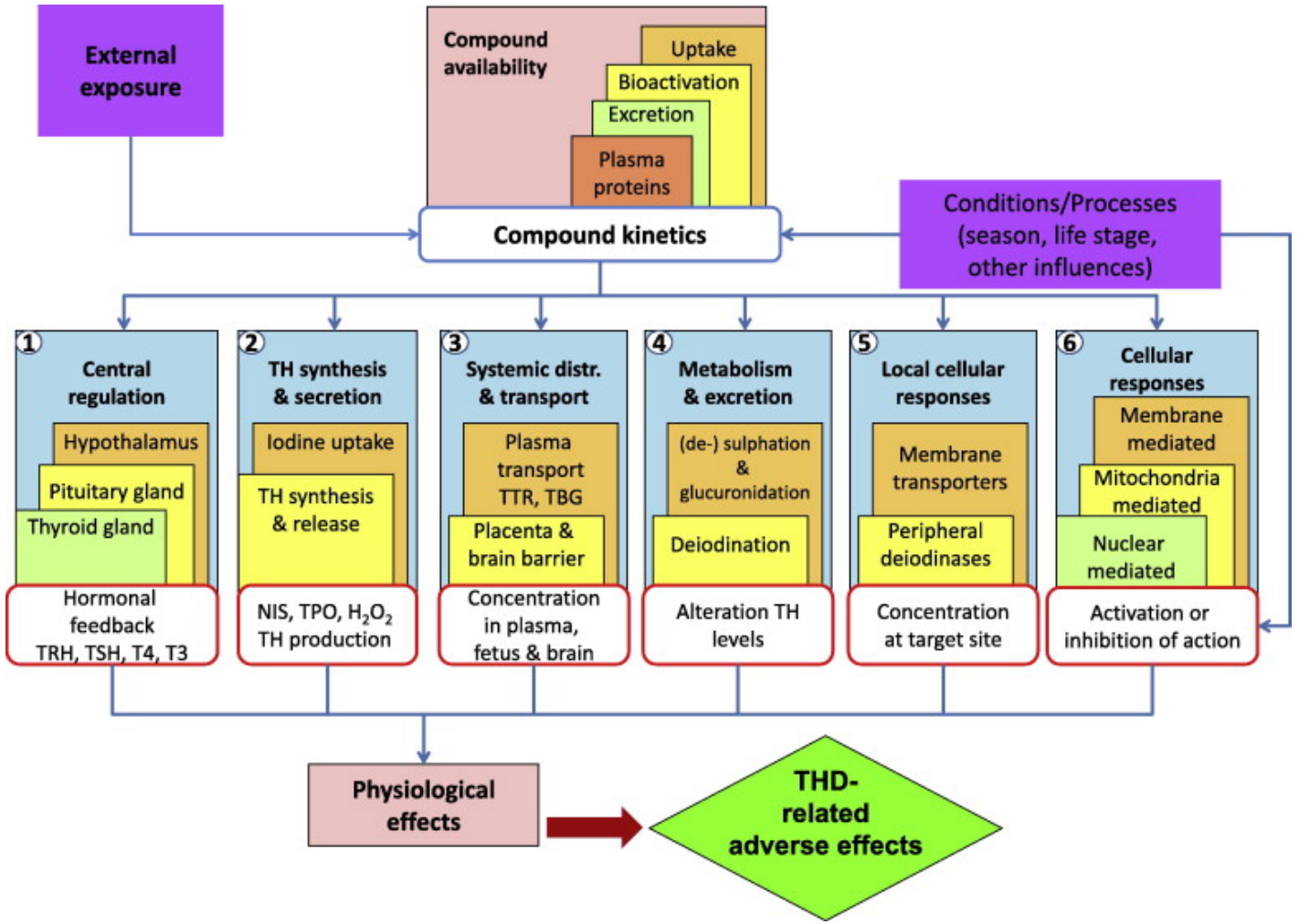
***In vivo* endpoints for thyroid-related endocrine testing in guideline studies**

- 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



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

2014 **OECD.** New Scoping Document on in vitro and ex vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling. *OECD Series on Testing and Assessment, No. 207*

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.*

DOI: 10.1016/j.tiv.2013.02.012

EU-NETVAL Validation of In Vitro Thyroid Test Methods

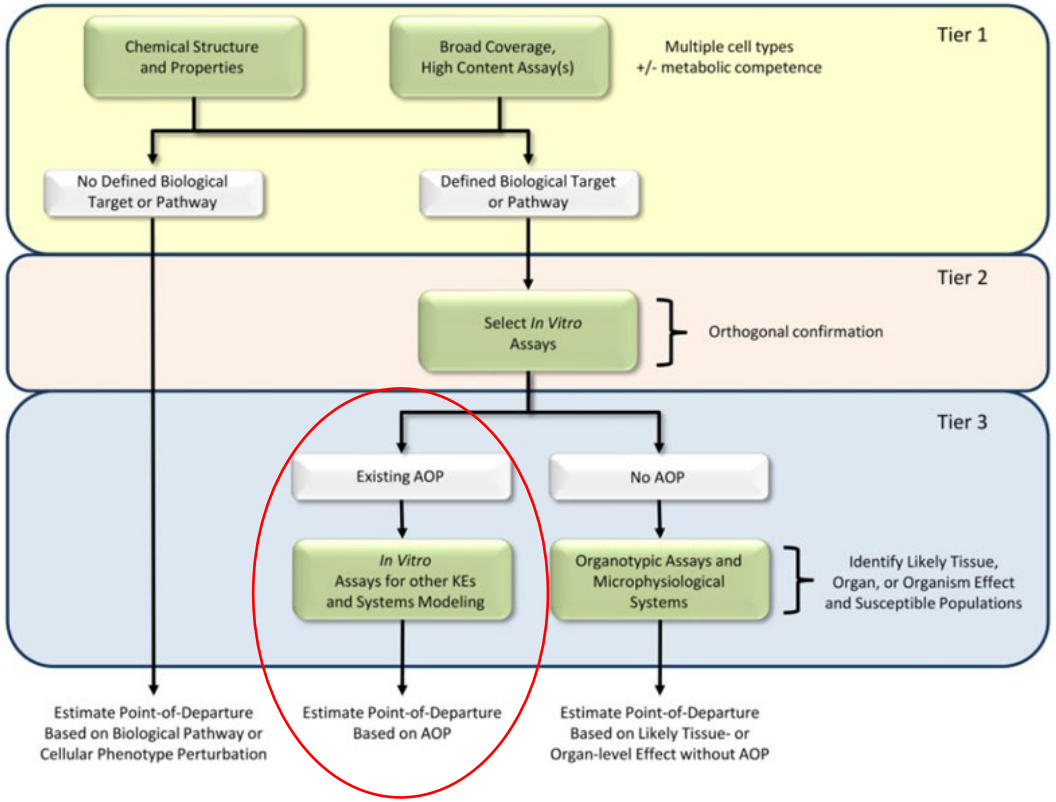
- EURL ECVAM has compiled a number of in vitro thyroid methods with validation potential based on OECD scoping document (OECD, No. 207, 2017)

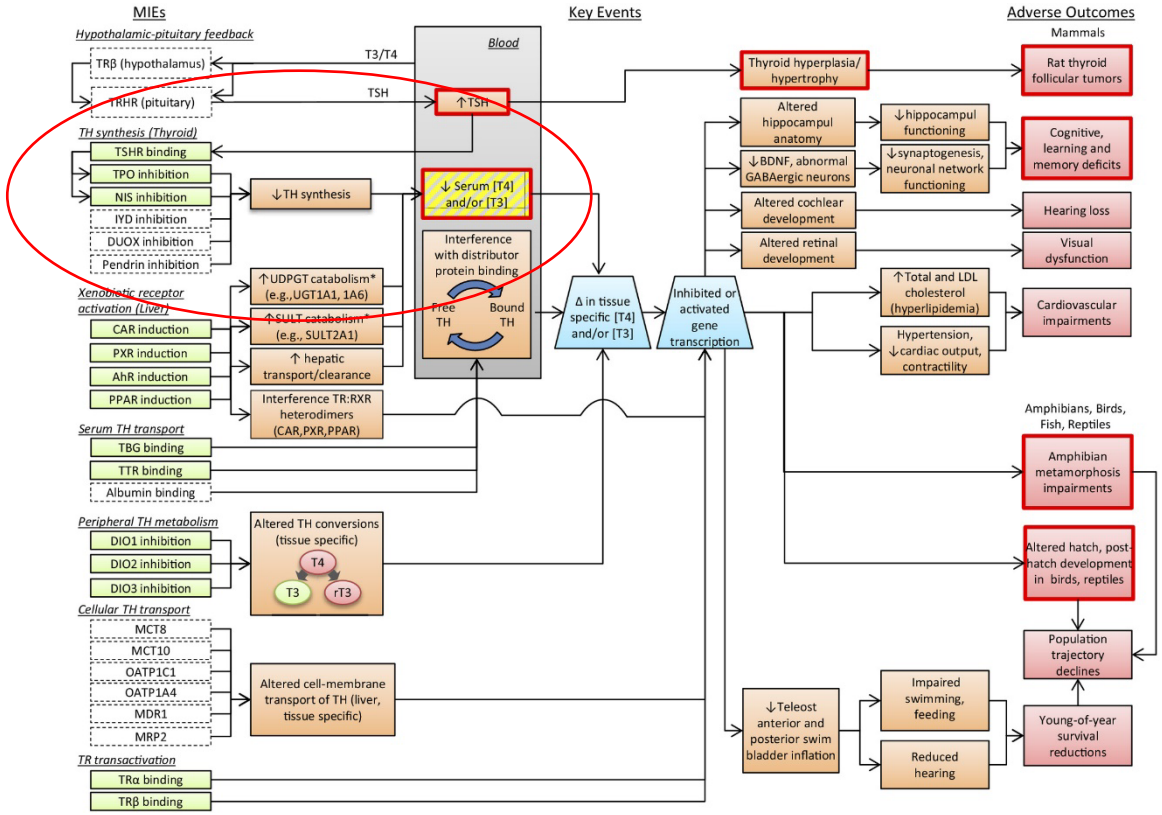
- European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) has 15 member labs participating in validation of 18 human-relevant mechanistic methods.

  - Block 1: Central Regulation
  - Block 2: Thyroid Hormone Synthesis
  - Block 3: Secretion and Transport
  - Block 4: Metabolism and Excretion
  - Block 5: Local Cellular Concentrations
  - Block 6: Cellular Responses
  - Block 7: Relevant Short Term Alternative Methods Integrating Multiple MOAs
  - Block 8: Integrative Cellular In Vitro Methods

- Final data collection anticipated for October 2022.

Challenges with *In Vitro* Thyroid Testing: Thyroid HTS Assays Do Not Directly Measure Thyroid Hormone Disruption

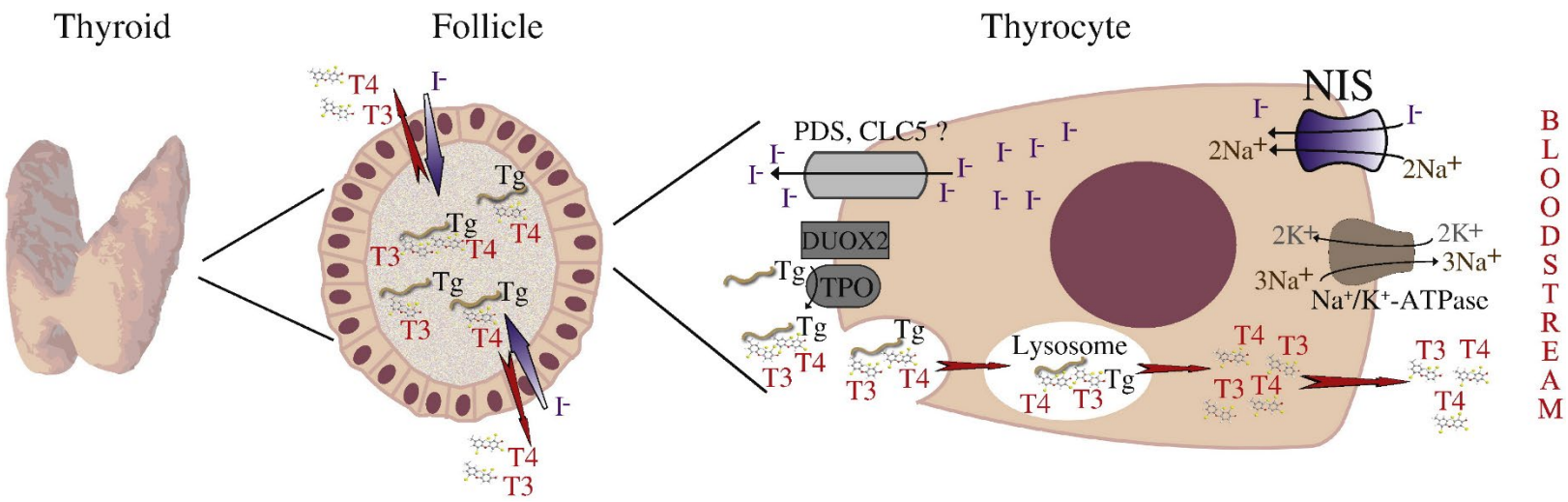




Target Gene	Assay	Environmental Chemicals Screened	Active Chemicals	% Active	Reference
TSHR	Engineered Cell Line	7871	825	10	TCPL: TOX21_TSHR_Agonist, TOX21_TSHR_Antagonist
TPO	Microsomal Enzyme	1074	314	29	K. Paul Friedman et al, ToxSci, 151(1), 2016, 160-180
NIS	Engineered Cell Line	293	137	47	J. Wang 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. Hornung 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	1815	148	8	J. Olker et al, Toxicol In Vitro. 2021 Mar;71:105073.

Thomas RS et al. Toxicol Sci. 2019;169(2):317–332; Noyes PD et al. EHP. 2019; 127(9):95001

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

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

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

TOXICOLOGICAL SCIENCES, 2019, 1–16
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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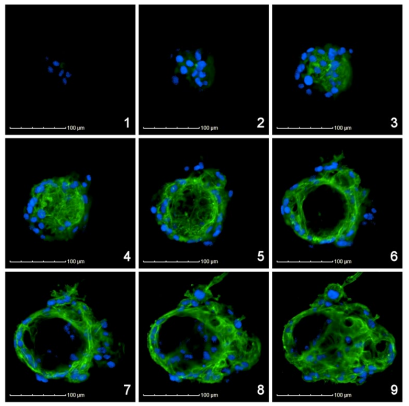
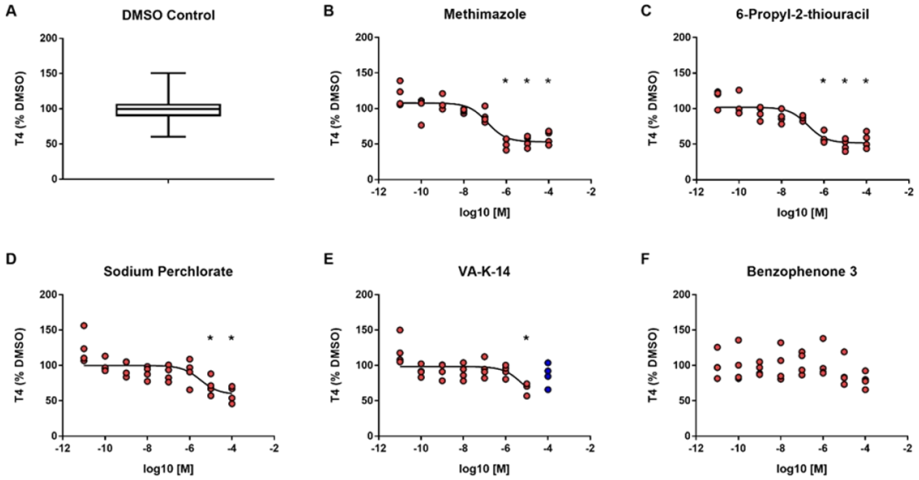




Challenge

- Thyroid high-throughput screening (HTS) assays do not directly measure thyroid hormone disruption.
- Many HTS prioritized chemicals need orthogonal confirmation for biological and mechanistic relevance.
- Regulatory decisions for chemical safety currently use apical endpoints like serum thyroid hormone levels as indicators of thyroid disruption.

Innovation

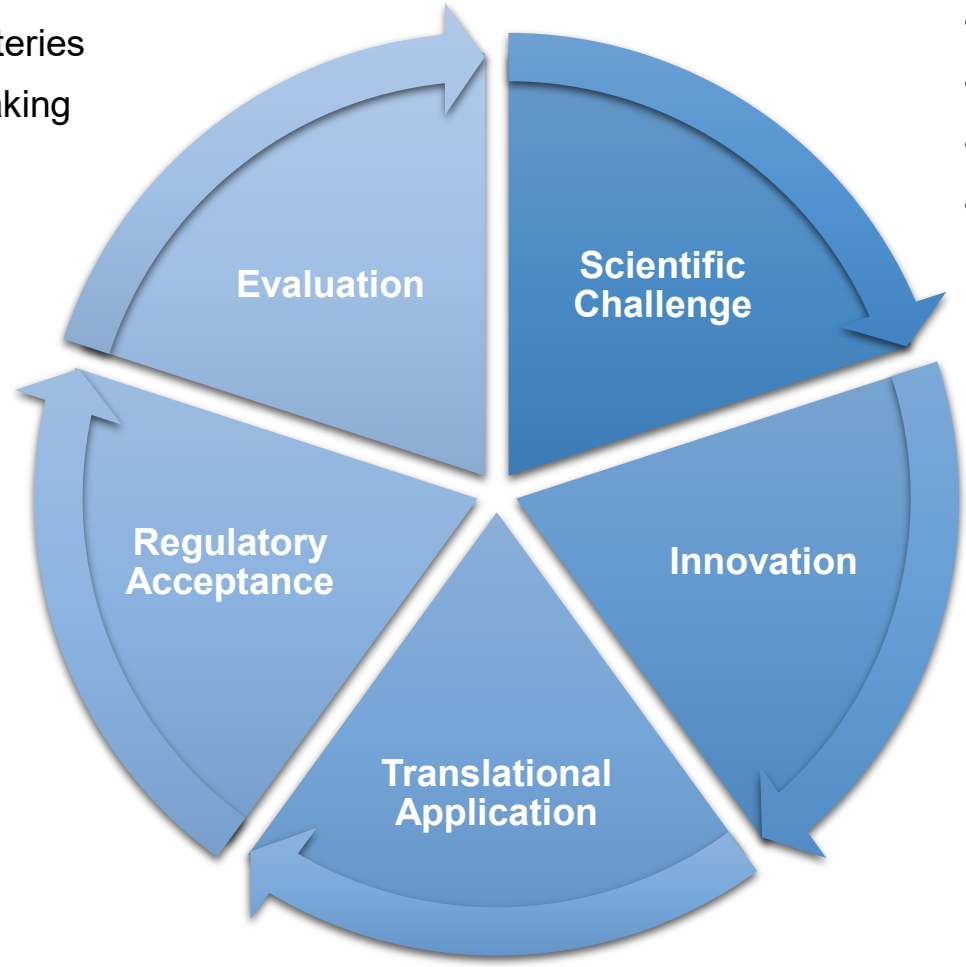
- Developed a human thyroid microtissue assay to evaluate chemical effects on thyroid hormone synthesis, secretion, and tissue viability.

Impact

- May enable chemical regulatory bodies to apply human in vitro data for identifying thyroid as a mode-of-action for endocrine disruption.
- Manufacturers of new chemical entities could benefit from insight into thyroid toxicity early in the development process.

In Vitro Test Method Life Cycle

- Defined problem
- Mechanistic reduction of biological system
- Scoping of extant methods and limitations
- Identification of priority needs



- Test method development, optimization, and improvement
- Proof-of-concept
- Performance evaluation

- Data generation for hazard identification and prioritization
- Data interpretation, interpolation, and extrapolation
- Novel biological and mechanistic insight

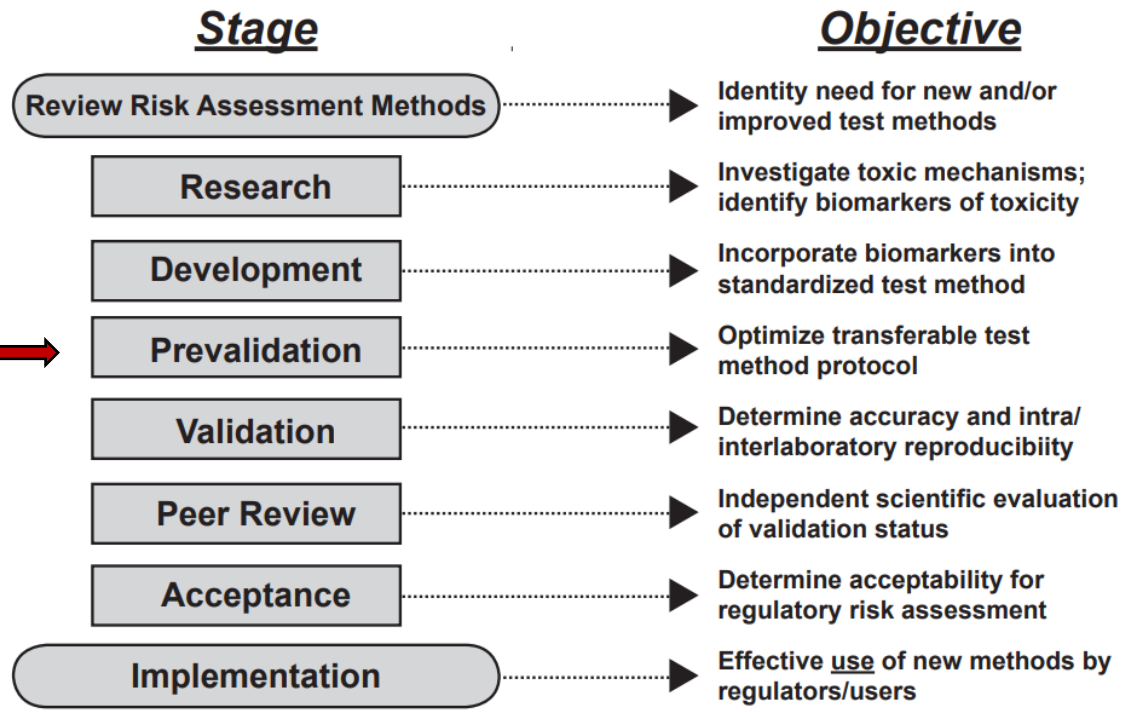
- Engagement of stakeholders and recognized validation authority
- Test method standardization
- Test method transfer
- Test method validation
- Test guideline adoption and implementation

- Defined approach test batteries
- Application to decision-making
- Utility evaluation
- New problem formulation

Test Method Validation Process

Most validation studies benefit from the process of prevalidation; a small inter-laboratory study conducted prior to the larger inter-laboratory validation study. (Curren, et al., 1995).

We are here. →



NICEATM-ICCVAM (NIH Publication No: 03-4508): ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods.

Thyroid Product Proposal:
Inter-laboratory Prevalidation Study of the Human Thyroid Microtissue Assay

Product Title: Inter-laboratory Prevalidation Study of the Human Thyroid Microtissue Assay.

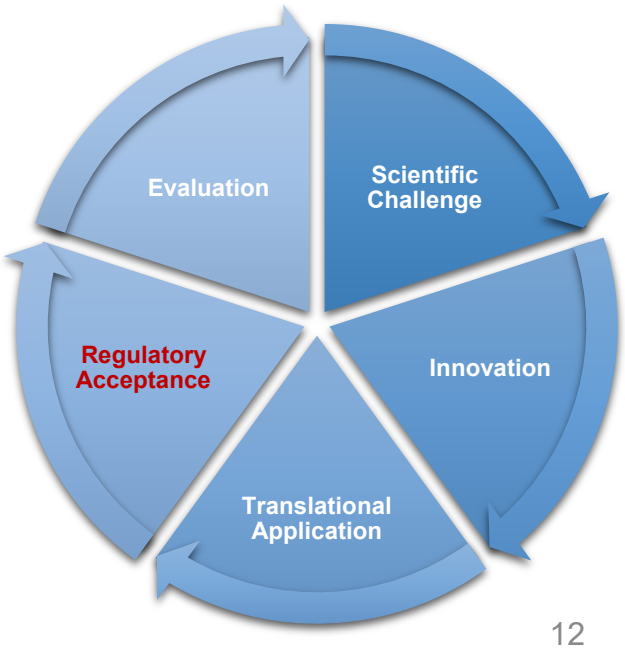
Primary Output: Chemical Safety for Sustainability - Virtual and Complex Tissue Models - 405.1

Partner Needs: Tiered testing strategies, Building confidence in new approach methods (NAMs), Vulnerable and sensitive life stages and subpopulations

Brief Description and Research Use: Validated new approach methods (NAMs) are needed to establish confidence and adoption to regulatory decision-making. The objective of this product is to conduct a small inter-laboratory prevalidation study to structure and support submission for a larger validation study. In collaboration with NICEATM-ICCVAM and partner laboratories, phased work will include protocol standardization, donor selection and quality control criteria, method transfer evaluation, and a blinded interlaboratory study with a set of reference chemicals. Successful completion would harmonize the NAM methods and facilitate advancement for consideration of a larger validation study by an internationally recognized validation authority.

Proposed Key Team Member Representatives

- Chad Deisenroth (U.S. EPA; Product Contact)
- Edward LeCluyse (LifeNet Health)
- Jessica LaRocca (Corteva Agrisciences)
- Nicole Kleinstreuer (NICEATM-ICCVAM)
- Program Partners (ICCVAM Agency Partners/OCSPP/OCHP)



Inter-laboratory Prevalidation of the 3D Human Thyroid Microtissue Assay

Goal: To structure and support a preliminary assessment of the test method performance and reproducibility for submission to a larger validation study.

Planning: Define and recruit appropriate stakeholders, laboratories and validation center representative to inform and implement study design.

Phase 1 - *Protocol Refinement*: Donor selection and acceptance criteria, technical optimization of the 3D microtissue assay.

Phase 2 - *Protocol Standardization*: Define standardized operating procedures.

Phase 3 - *Protocol Transfer*: Method transfer evaluation.

Phase 4 - *Protocol Performance*: Conduct a blinded interlaboratory study.

Phase 1 - Protocol Refinement: Donor selection and acceptance criteria, technical optimization of the 3D microtissue assay.

Objective: RVA, Lab 1, and Lab 2 will participate in the creation of the overall study design, analytical approaches, and data interpretation for the optimization of the human thyrocyte model system within their respective capabilities and expertise.

Definitions

- Recognized Validation Authority (RVA): NICEATM – ICCVAM
- Lab 1: EPA ORD/CCTE – Deisenroth lab
- Lab 2: LifeNet Health (LNH)
- Lab 3: Corteva Agrisciences

1. Develop a minimum characterization strategy and acceptance criteria for qualifying suitable batches of primary human thyrocytes for testing purposes*.

2. Determine optimal culture conditions, medium formulation, time points, endpoints, and analytical methods for developing a final chemical testing protocol.

3. Establish a suitable reference compound set representing different modes-of-action and their optimal dosing ranges, exposure frequency, duration and endpoint analysis for the assay.

* Use of primary cells in the assay presents additional challenges for validation.

Donor Qualification: Establishing a COA for Primary Thyrocytes

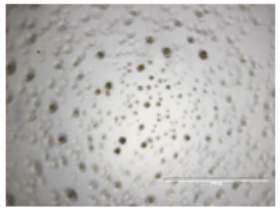
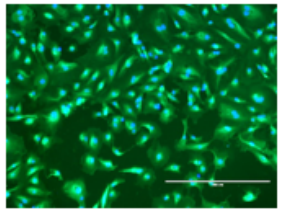
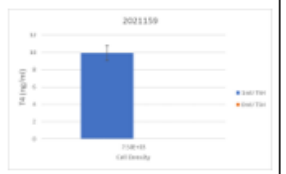
LifeNet Health LifeSciences Division

CERTIFICATE OF ANALYSIS DRAFT

CELL TYPE	Primary Human Thyrocytes		
Product Code		Lot No	THY2021159
Thyroid Disorder	Negative	Passage Number	P1
Storage Condition:	Cryopreserved in Vapor Phase Liquid Nitrogen (< -150°C)		

DONOR DEMOGRAPHICS								
Age	Sex	Race	BMI	Tobacco Use	Alcohol Use	Drug Use	Medication Use	Cause of Death
43	M	C	23.4	None	None	None	Azelastine HCL	CNS Tumor

QUALITY TEST	SPECIFICATION	RESULT
Morphology	Microtissues; Follicle-like morphology (≥50µm)	(Attached image)
Viable Cell Count	≥ 0.5 x 10^6 viable cells per vial	0.33 x 10^6 viable cells
Viability	≥ 75% post thaw	Pass
Plateability	14 days	Pass
Purity (Flow Cytometry)	TBD TG	(ND)
	TBD TSHR	(ND)
Purity (ICC)	Positive: ≥ 90% KRT7; 53% TG	Pass
Functionality (1mU/ml TSH)	≥2000ng/ml TG; Day 7 (1.5 x 10^4cells/well)	Fail
	≥3ng/ml T4; Day 14 (1.5 x 10^4cells/well)	Pass
Virus	Negative: HIV-1, Hepatitis-B, and Hepatitis-C	Pass
Sterility	Negative: bacteria, yeast, and fungi	(ND)

MORPHOLOGY (10x)	ICC (TG) 10x	TG ELISA	T_4 ELISA
			

Phase 2 – Protocol Refinement: Define Standardized Operating Procedures.

Objective: Lab 1, and Lab 2 will participate in developing and drafting of standardized test procedures for the human thyroid microtissue assay. Documentation of the test method and state of readiness will be guided by the following elements as defined in OECD Guidance Document on Good In Vitro Method Practices (GIVIMP) and accompanying templates for cell-based toxicological assays.

OECD Guidance Document on Good In Vitro Method Practices

- Roles and responsibilities
- Quality considerations
- Facilities
- Apparatus, material and reagents
- Test systems
- Test and reference/control items
- Standard operating procedures
- Performance of the method
- Reporting of results
- Storage and retention of records and materials

1. Create GLP-compliant standard operating protocols (SOPs) integrating guidance from OECD Guidance Documents on Good Cell Culture Practice (GCCP) 2.0 and Good In Vitro Method Practices (GIVIMP) to ensure sound scientific, technical, and quality practices.
2. Determine intra-laboratory reproducibility of the method using matched donor materials in Lab 1 and Lab 2.
3. Evaluate suitability for Phase 3.

Phase 3 – Protocol Transfer: Method Transfer Evaluation.

Objective: Lab 3 will transfer and execute standardized operating protocols for intra-laboratory validation of the human thyroid microtissue assay.

- The availability, diversity, and number of human donors will be established to adequately address variability and performance criteria.
- Assays will be conducted in strict concordance with defined test methods.
- Monitor quality assurance/quality control of method transfer and execution.
- Evaluate established assay performance and reproducibility.

1. Transfer the SOPs to Lab 3.
2. Determine intra-laboratory assay performance and reproducibility in Lab 3 using matched donor materials from Phase 2.
3. Refine SOPs, as necessary.
4. Evaluate suitability for Phase 4.

Phase 4 – Protocol Performance: Conduct Inter-laboratory Validation.

Objective: Lab 1, Lab 2, and Lab 3 will contribute to the execution of standardized test protocols for inter-laboratory validation of the human thyroid microtissue assay.

- The availability, diversity, and number of human donors will be established to adequately address variability and performance criteria established in Phase 1-3.
- Assays will be conducted in strict concordance with defined test methods.
- Monitor quality assurance/quality control of method transfer and execution.
- Evaluate established assay performance metrics for sensitivity, specificity and reproducibility.

1. Define the aim(s) of Phase 4 study, using adjustments of outcomes from Phase 1-3.
2. Test a set of blinded chemicals using the final SOPs.
3. Report outcome in a joint peer-review publication detailing the scope of work and analysis of test data.
4. Review performance of the method based on study outcome and evaluate for subsequent action.

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Jeff Thomas
Jingsong Chen
Mary Jane Cunningham
Paul Gallant
Rony Thomas

Jessica LaRocca
Enrica Bianchi
Mercedes Biven

Supplementary: SOPs

Category	Method
Medium	Preparation of Humanized 7 Hormone (h7H) Medium Formulation
Cell isolation	Primary Human Thyroid Cell Isolation
Cell recovery and maintenance	Primary Human Thyroid Cell Maintenance and Passaging
Cryopreservation and storage	Primary Human Thyroid Cell Cryopreservation
Thawing and plating	Plating Procedure for Cryopreserved Primary Human Thyroid Cells
Pathogen testing	Pathogen Testing of Cryopreserved Primary Human Thyroid Cells
Biomarker profiling/purity	Immunocytochemistry of Primary Human Thyroid Cells in a 2D Culture Model
Biomarker profiling/purity	High Content Image Analysis for Human Donor Thyroid Cells - KRT7, TG, NKX2.1
Biomarker profiling/purity	Flow Cytometry Analysis for Human Donor Thyroid Cells - TG, TSHR
Assay	3D Culture Model of Primary Human Thyroid Cells
Endpoint Analysis	Thyroglobulin (TG) ELISA
Endpoint Analysis	Thyroxine (T4) Competitive ELISA
Endpoint Analysis	Thyroxine (T4) LC-MS/MS
Endpoint Analysis	Triiodothyronine (T3) Competitive ELISA
Endpoint Analysis	Triiodothyronine (T3) LC-MS/MS
Viability Analysis	CellTiter-Glo Viability Assay in Primary Human Thyroid Cells