Co-Aegis – *Concept and Exploratory Validation Roadmap*
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1 Clinical Gap

Checkpoint-refractory solid tumours lack a one-shot curative option. A therapy that **destroys hypoxic cores** *and* **primes systemic immunity** would fill that void.

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2 Proposed Solution

Inject **dormant *Clostridium novyi-NT*** (Δα-toxin) spores that

- **Wake only at ≤ 1 % O_2** inside tumour zones.
- **Lyse tumour cells in-situ** → neo-antigen flood.
- **Shut off on command** via doxycycline-triggered self-kill ± standard IV antibiotics.

This native lytic effect functions as the default payload: once spores germinate, vegetative *C. novyi-NT* disrupts tumour cells mechanically and enzymatically, creating necrosis without requiring an engineered toxin.

Strain background ATCC BAA-3311 / DSM 19523 · genome CP023719 · natural plasmid Δα-toxin · uracil-auxotroph.
Heat-shock 80 °C × 10 min removes vegetative cells; spore yield ≈ 3×10^{11} L^{-1}.

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3 Containment & Recovery Architecture

The Co-Aegis chassis applies layered logic to ensure tumour-restricted activity, passive safety, and diagnosable failure modes. Each layer is either self-limiting or reversible.

Layer	Type	Function	Spec target
Oxygen gate	Passive	FNR-promoter controls *spo0A* germination gene	No germination > 1 % O_2 (leak ≤ 10^{-5})

L-dAP metabolic addiction	Passive	Plasmid loss → no DapA → death within ~4 h	Composite escape ≤ 10^{-9}
Suicide ori (RCR–)	Passive	Prevents plasmid retention or HGT	Escape ≤ 10^{-9} (Goodman 2023)
Integrity exposure system	Passive-reactive	Deviation from core logic triggers surface phosphatidylserine mimic	Enables silent local clearance via efferocytosis (immune-independent)
Clinical abort switch (Cas9 + doxycycline)	Active-optional	200 mg IV doxy triggers Cas9-based self-lysis	≥ 4-log CFU drop · T½ ≤ 45 min

System framing:

- **Passive logic** ensures dormant spores cannot activate outside pathologically hypoxic cores (e.g. tumour microenvironments). While targeting does not discriminate tumour tissue per se, off-target zones (e.g., ischemic ulcers) remain subject to immune clearance via the **Exposure Module**.

- During dormancy, Co-Aegis expresses a minimal surface-layer **Harmonizer Peptide**, derived from commensal biointerfaces, to reduce immune friction and environmental disturbance. It conveys inertness, not stealth — and degrades upon germination or design failure.

- If key design features are lost (e.g. auxotrophy, germination logic), the **Integrity Exposure Module** reveals cells to the immune system.

  Upon core logic failure, Co-Aegis passively surfaces a phosphatidylserine mimic (PSM) for silent clearance via local efferocytosis, and emits a diagnostic metabolite trace (TMR) that identifies the failure mode non-invasively.

- **Cas9-based shutdown** is retained for active clinical abort.

- **Hypoxia-gated germination** is refined to require not only O_2 ≤ 1 % but sustained hypoxic descent (e.g., ≥12 minutes at ≤1.2 %, or ≥4 minutes at ≤0.5 %), minimizing activation in unstable tumour margins. This is implemented via a tuned *Pfnr*-regulated *spo0A* promoter with upstream RNA-level persistence gating (toehold logic).

- **Optional PET imaging module**: silent HSV-TKmut under hypoxia promoter allows on-demand ^{18}F-FHBG scans to confirm in-vivo germination.

- **Antibiotic stewardship**: MIC-shift monitoring in Step 2; fallback macrolide pathway predefined.

3a Payload Flexibility (Chassis Modularity)

The Co-Aegis construct allows the Cas9 cassette to be replaced with any therapeutic payload without altering the containment logic.

All payload modules are required to remain within the defined Payload Compatibility Envelope (PCE). This includes limits on transcriptional and translational burden (e.g., mRNA size ≤ 2.5 kb, protein synthesis cost < 15% host ATP budget), immune non-interference (e.g., no shielding of FLAG or quorum output), and metabolic inertness outside of induced states. Payloads exceeding these boundaries may compromise system balance and must undergo separate integration validation.

Payload Type	Use-case	Control logic
PD-L1 nanobody	Local immune checkpoint blockade	Tet-inducible
GM-CSF + IL-12 (bicistronic)	In-situ tumour vaccine	Tet-inducible
Collagenase + TGF-β trap	Stroma remodelling (desmoplastic tumours)	Tet-inducible
IL-10 microburst (Step-2 only)	Inflammatory dampener (auto-trigger if IL-1β > spec)	Quorum + hypoxia gated

All payloads are gated by the same regulatory switch logic and remain dormant unless spores germinate in hypoxia. Cas9 remains the default cassette in Phase Ia.

3b Integrity Exposure System

To address mutation, plasmid loss, or circuit breakdown, Co-Aegis includes an **autonomous integrity-sensing module**.

If the design fails structurally — e.g., due to silencing of spo0A, dapA, or Cas9 (toehold logic) — the construct self-tags for clearance via passive apoptotic mimicry:

`[Pfault] → sigPep-PSM → T5`

This module expresses a phosphatidylserine mimic on the cell surface, enabling silent clearance via local efferocytosis pathways.

Clearance is immune-independent and remains functional even in immunosuppressed hosts. No adaptive priming is required.

This exposure response requires no clinician input. It is **self-activating**, **localized**, and **non-lethal to the system** unless external clearance is triggered.

If paired with a companion biologic (e.g., anti-FLAG nanobody or $CD8^+$ primer), failure states become self-resolving without impacting dormant spores.

An optional Trace Metabolite Reporter (TMR) module is linked to Pfault. Upon logic failure (e.g., loss of spo0A, dapA, or Cas9), the system emits a kidney-clearable, non-bioactive reporter metabolite. This fragment correlates with the failure mode (e.g., spo0A dropout = TMR-01), enabling downstream non-invasive trace detection (e.g., urine ELISA or LC-MS). TMR emission is orthogonal to immune exposure and PET signal, and functions even in immunosuppressed contexts. Fragments are <2 kDa and tagged with a trace-resolved barcode or peptide sequence. Clearance is complete within 24–48 h post-trigger.

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4 Indicative Budget & Timeline (Steps 1–2)

Primary Route – In Vivo Readout

Work-package	€ k
Genome editing & verification	35
Oxygen-gate & abort logic assays	25
Rodent pilot (necrosis + safety)	135
Contingency (10 %)	20

Total (≈ 9–11 months)	215

Figures are order-of-magnitude estimates to guide planning.

Alternative Route – Minimal Loopback Platform (In Vitro)

Co-Aegis preclinical validation may alternatively proceed via a non-sentient system:

- 3D tumour spheroids perfused under hypoxia
- Reporter signal tracking (PET analogue or fluorescence)
- PBMC loopback co-culture for CD86↑, AH1-tetramer $CD8^+$ T cells, and IFN-γ ELISPOT

This platform preserves all key immunological readouts while reducing procedural and ethical complexity.

Work-package	€ k
Spheroid chamber setup & validation	7
Reporter signal & necrosis assay	3
PBMC co-culture + immune profiling	10
Contingency	2
Total (matched validation window)	22

This route may reduce operational scale while retaining the fidelity of adaptive immunity readout.

5 Evidence Ladder – Structural Validation Path

Validation is not a singular event, but a sequence of confirmable structure–response relationships. Each step confirms that Co-Aegis behaves as designed — within architecture, not assumption.

Step	Key assays	Success threshold	Effort
1 In-vitro feasibility	O_2-gradient germination · doxycycline abort-curve	Meets §3 containment specs	≈ 3 mo · €65 k
1b PBPK modelling	PK model (plasma → liver → tumour → brain → gut) using reference doxy parameters	IC_{90} in tumour ≤ 30 min · brain ≤ 45 min · abort window ≥ 2 h	≈ 1 wk · €5 k
2 Rodent pilot/MLP	CT26/MC38 intratumour dose → MRI necrosis · blood CFU · rescue window ± doxy PK	≥ 30 % necrotic core @ d7 · blood CFU ≤ 10 @ 24 h · IC_{90} confirmed in situ	≈ 6 mo · €160 k

Clean-Lysis & Adaptive-Immunity Submodule (embedded in Step 2)

Metric	Assay	Target
LPS load	LAL	< 0.5 EU mL^{-1} @ 24 h
PGN / CpG debris	LC-MS + IL-1β ELISA	within physiological tolerance
Bystander fibroblast viability	Co-culture 24 h	≥ 90 %
Innate activation	CD86↑ on tumour DCs (d3)	≥ 2× baseline

Adaptive priming	AH1-tetramer CD8 % (d7)	≥ 0.3 %
IFN-γ ELISPOT	Splenocytes (d7)	≥ 150 SFC / 10^6
Functional memory	Tumour re-challenge (d28)	≥ 50 % rejection

Inflammation dampening trigger (auto-fire IL-10 cassette):

> *Fires only if IL-1β or TNF-α > ceiling → Returns IL-1β to baseline ≤ 6 h*
> *CD86↑ remains ≥ 2× baseline throughout*

Cost increment for immunologic dampening validation: ≈ €15 k · timeline + 2 weeks

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Integrated Trace Layer: Self-Attesting System State (SASS)

Step 2 includes generation of a **verifiable telemetry signature**, confirming real-time performance against architectural thresholds:

- Germination latency vs hypoxia persistence
- PET trace coupling to spo0A activation
- Abort logic execution window
- Plasmid retention check under stress
- exposure fidelity via Pfault trigger logic
- MIC curve shift detection via ART telemetry
- Evidence Ladder versioning and construct ID binding

Each validated construct emits a **traceable system fingerprint** — cryptographically stable, locally loggable, and transferable across institutional settings.

This enables downstream teams to **verify structural equivalence without full assay repetition**, provided context and regulatory class match. The system is not trusted — it is *testable again by design, or verified by trace.*

> *Note: Signatures remain valid only when architecture, genomic integrity, and environmental class are conserved. Drift or payload swap invalidates fingerprint and requires fresh validation.*

The TMR module extends the self-attesting signature logic to include non-imaging, non-immune failure mode logging. If Co-Aegis self-exposes via Pfault, a corresponding metabolite fragment is emitted into circulation. This trace remains detectable in urine or saliva for 24–48 h and corresponds to a unique failure code (e.g., FM01 = spo0A silencing). Detection enables passive post-resolution diagnosis, even in absence of PET or immune interaction.

Total Base Validation Path

Includes in-vitro, PK, rodent, immunologic, and self-attesting telemetry
Figures represent structurally realistic, comfort-level estimates — not grant-locked budgets.

Estimated consolidated effort: ~9–11 months
Estimated base cost (incl. trace infrastructure): ≈ €245–250 k

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6 Existing Evidence (Key Literature)

1. **Phase I human (n = 24):** 42 % major necrosis *(Janku 2016)*
2. **Client-dog sarcoma (~200):** 19 % complete responses *(Dang 2024)*
3. **Murine CT26 ± PD-1:** 2× tumour-free survival *(Guan 2022)*
4. **Genome CP023719:** no residual toxin ORFs *(NovyiRef 2018)*
5. **Tet-Cas9 kill-switch:** 4.3-log kill ≤ 90 min *(Shen 2022)*
6. **Suicide-ori HGT containment:** escape ≤ 10^{-9} *(Goodman Nat Biotech 2023)*
7. **Doxy tumour PK:** IC_{90} reached ≤ 30 min *(Luo J Pharm Sci 2021)*
8. **L-dAP metabolic addiction:** zero revertants 30-day passage *(Wu Nat Chem Biol 2024)*

(Full reference list available on request.)

7 Platform Outlook

- Beyond tumour lysis, the chassis **may address chronic inflammation and health-span extension** once configured for low-dose immune-modulation. This therapeutic horizon is not part of the current validation, but informs long-range design.

- IV formulations could accelerate tumour deposition through
  (i) a transient hypoxia-enhancing pre-dose or
  (ii) ultrasound-triggered microbubble release; both require no genetic modification of the Co-Aegis chassis.

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8 Suggested Roles (Structure-Linked)

The following functions support safe, trackable implementation of the Co-Aegis system. Roles may be merged or distributed depending on institutional structure.

Function	Structural Responsibility
Anaerobe system lead	Execute germination assays, manage spore culture and oxygen gating validation (Step 1)
Preclinical validation lead	Oversee necrosis readout and immune profiling in chosen platform (rodent or in-vitro loopback)
Containment & rescue logic adviser	Define antibiotic SOPs, monitor plasmid loss and abort-path PK (linked to §3 layers)
Regulatory interface	Map contained-use requirements, dossier format, and ethical review path for chosen readout tier
Payload logic designer *(optional)*	Support modular cassette swaps (e.g. IL-10, PD-L1, IL-33) under existing control logic

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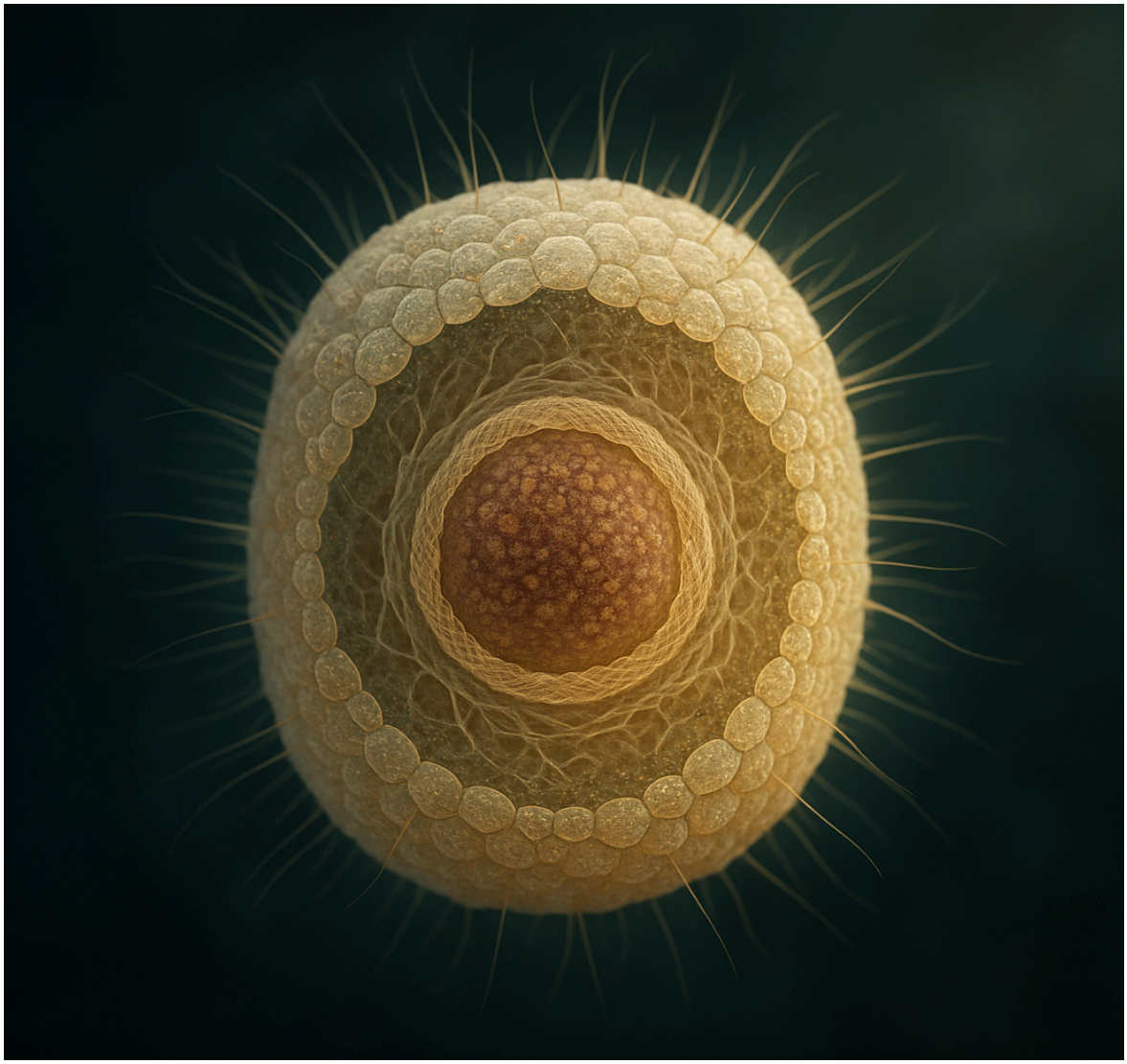
Figure A1. Structural Depiction of Co-Aegis Chassis in Dormant State

Description:

High-fidelity digital rendering of the Co-Aegis therapeutic system in its pre-germination (dormant) state. The image depicts the full containment stack as structurally inert: hypoxia-gated germination logic (spo0A under Pfnr) remains unexpressed; the metabolic addiction layer (dapA complementation plasmid) is held passively; abort and payload logic (e.g., Cas9 cassette) remain transcriptionally silent. Fine external filaments symbolize latent environmental sensing (e.g., oxygen

or quorum gradients), while the inner bracketing layers reflect complete architectural closure. No integrity exposure signal is present — indicating absence of deviation or logic failure.

The illustration conveys the chassis as a conditionally inert structure — fully formed, structurally honest, and awaiting biochemical invitation.



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